Advances in Molecular Toxicology

Volume 2

VOLUME TWO

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VOLUME TWO

ADVANCES IN MOLECULAR TOXICOLOGY

Editor

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Editor's Preface

This second volume of Advances in Molecular Toxicology, like its predecessor, does not dwell on a particular focus. Instead, the editor has invited high quality contributions from among the field's top practitioners to highlight the diversity of interests, areas and approaches encountered in this discipline. It is hoped that in perusing the enclosed chapters, the reader will readily appreciate the highly interdisciplinary nature of the research in these areas. While this may seem daunting to the new practitioner or beginning student, it should surely be exciting to recognize that recent advances in these and related areas draw on the skill sets of the many disciplines for which molecular toxicology is an interface.

Owing to a historical lack of prudent waste management practices, chromate is unfortunately extensively dispersed in the human environment due to the use of chromium in manufacturing. There is an ongoing workplace exposure as well. Chromate has long been known as a genotoxin. Despite this, the molecular basis of its genotoxicity remains a matter of contention. This is partly due to multiple redox states and also the somewhat perplexing spectrum of chemistries in which some of these states can participates. James Covino and Kent Sugden elaborate the situation admirably in Chapter 1.

Toxicity in the liver, hepatotoxicity, is a major barrier in the development of new drugs and a leading cause of post-marketing withdrawal of pharmaceuticals. Rosiglitazone, an anti-diabetic, and nefazodone, an anti-depressant, are examples of entities withdrawn from market due to hepatotoxicity for which structural analogues with better safety profiles have remained on market. Examination of the detailed metabolism of withdrawn and still-marketed analogues is the subject of Axel Pähler and Christoph Funk's review in Chapter 2. This comparative approach can reveal the sources of particularly toxic functionalities and also allow the development of some predictive power for avoiding/reducing hepatotoxicity and aiding in development of safe new therapeutics.

Bennedetta Sallustio elaborates the 'dark side' of glucuronide conjugates in Chapter 3. Historically such conjugates of xenobiotics and their metabolites were considered safely destined for export. But more recent work has detailed greater complexity. The glucuronide functionality targets certain tissues due to specific transporter systems and can chemically activate certain functional groups or cause damage by means of the potential inherent in the glucuronide functionality. The chemistry and biochemistry of a number of recent examples is detailed within.

Allergic contact dermatitis affects a significant portion of the population and derives from contact with agents as simple as metal ions to molecules such as fragrance constituents. The molecular basis for this reaction is only recently becoming clear. Initiation events involve interaction with or "haptenization" of proteins, in the latter case often subsequent to metabolic activation. And the ultimate manifestation is immune-mediated. The complex interplay of the

chemistries of sensitization and biology of elicitation, as well as the predictive potential, based on the spectrum of agents known to elicit contact dermatitis, is the subject of Chapter 4 authored by Ann-Therese Karlberg, Jens Baron and Hans Merk.

The stimulating complexity of the toxicology of metals and metalloids is the subject of Chapter 5 authored by Graham George, Ingrid Pickering, Christian Doonan, Malgorzata Korbas, Satya Singh and Ruth Hoffmeyer. Arsenic and mercury are the prototypical elements that illustrate the considerations. A key aspect in toxicity of metals is molecular form as this dictates chemistry, uptake and distribution. A highlight of this chapter is the application of powerful tools for elucidating structure including variations of X-ray absorption spectroscopy and computational methods. Ultimately these approaches and their conclusions bear on therapeutic approaches to heavy metal toxicities.

Agus Darwanto, Lynda Ngo and Lawrence Sowers thoroughly review the literature on pyrimidine DNA base damage and repair in Chapter 6. This review takes a sweeping approach from the chemical elements to larger biological implications. Particularly intriguing is the novel hypothesis, pioneered by the principal investigator, that some types of damage, endogenously originated halogenation from immune response, may result in epigenetic changes devolving as altered gene expression with toxic consequences. This is potentially a long sought link between activated immune response and toxic consequences, clearly an area that will attract significant future attention.

The final chapter in this volume, Chapter 7, summarizes key aspects of DNA damage by exogenously encountered toxins. It emphasizes often under-appreciated aspects that contribute to ultimate biological consequences such as adduct persistence in the context of available repair mechanisms. The review also highlights the important contribution of polymorphism in xenobiotic metabolism and DNA damage repair to the ultimate impact of xenobiotic assault. These factors are important contributors to inter-individual variability in response. Annie Pfohl-Leszkowicz has eloquently summarized these key contributors that mediate DNA damage and its consequences.

This volume is dedicated to Chris Michejda and Bob Moschel, both of whose careers were sadly cut short by untimely demise. Both men's early professional careers were rooted in fundamental aspects of the chemistry of toxins. From these strong roots both evolved understandings and approaches that allowed them to craft important contributions to human health. Both were leaders in their fields and all who encountered them were struck by their selfless generosity. The editor gratefully recalls that each welcomed him to the discipline and gave him tangible encouragement. For he and many others, there is a palpable and inextinguishable void.

CHAPTER 1

Genotoxicity of Chromate

James J. Covino *and* Kent D. Sugden*

Contents

1. Introduction

The hexavalent oxidation state of chromium, chromate or Cr(VI), has been established as a human respiratory carcinogen based on more than a century's worth of epidemiological and medical evidence. Several million people worldwide are occupationally exposed to chromate and many more are at risk of environmental exposure arising from chromate contaminated landfills and aquifers. Over the years, research has illustrated chromate's myriad mutagenic, genotoxic, and carcinogenic effects in a wide range of *in vitro*, cellular, and whole animal model systems. However, the exact nature of the lesion(s) responsible for chromium's toxic and mutagenic effects has yet to be elucidated. The lack of clear mechanistic information regarding chromate genotoxicity is, at least partly, due to the number of different oxidation states of this metal that may play a role in its carcinogenicity, and the multiple oxidation and binding pathways that have been proposed to account for the wide assortment of DNA lesions observed in cellular systems.

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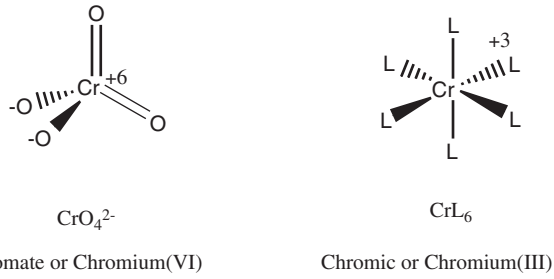
Little is known about the structural basis of these lesions. Recent advances in the field have begun to shed light on the mode of action at the molecular level for this metal. This review deals with current progress on our understanding of the interaction of chromium with DNA and should help to elucidate the mechanism of chromate-induced genotoxicity.

2. Chromate Uptake, Metabolism and Speciation

The toxic effects of soluble Cr(VI) are a direct consequence of its aqueous geometry, charge, and redox properties at physiological pH. The end product of intracellular chromate metabolism is the octahedral, +3 oxidation state, Cr(III), (Figure 1). Cr(III) complexes with certain ligands are toxic, mutagenic, and clastogenic [1–6] but the carcinogenic risk of this oxidation state to humans has been considered inconclusive by the Agency for Toxic Substances and Disease Registry (ATSDR) [7] and the International Agency for Research on Cancer (IARC) [8].

The cellular uptake and metabolism of chromate are described by the classical uptake-reduction model [9]. Hexavalent chromium, being isostructural with phosphate and sulfate, allows for rapid, and active, uptake through nonselective anionic membrane channels. Once internalized, chromate is rapidly reduced to Cr(III) by endogenous reductants resulting in the unidirectional accumulation of chromium in the cell. Accumulations of intracellular chromium at concentrations greater than 1 mM have been observed in cell culture following a 10 μM chromate exposure in the extracellular media [10].

In vitro studies have shown that many different intracellular constituents and organelles possess the ability to reduce chromate including microsomes [11,12], mitochondria [13], hydrogen peroxide [14], NADPH [15], ascorbate [16–20], glutathione [21–24], and cysteine [23,25]. Of these, ascorbic acid and glutathione are considered to be the major cellular reductants of Cr(VI) due to their relative reduction activity and their high cellular abundance [20]. While ascorbate and glutathione are present in roughly equimolar concentrations in the cell, ascorbate reduction was observed to be 61 times faster than that of glutathione [20]. In addition, 80% of chromate reduction in kidney, liver, and lung has been attributed to ascorbic acid metabolism [26,27]. Based on these and a number of other studies,



CrO_4^{2-}

Chromate or Chromium(VI)

CrL_6

Chromic or Chromium(III)

Figure 1 Structures of the two stable oxidation states of chromium.

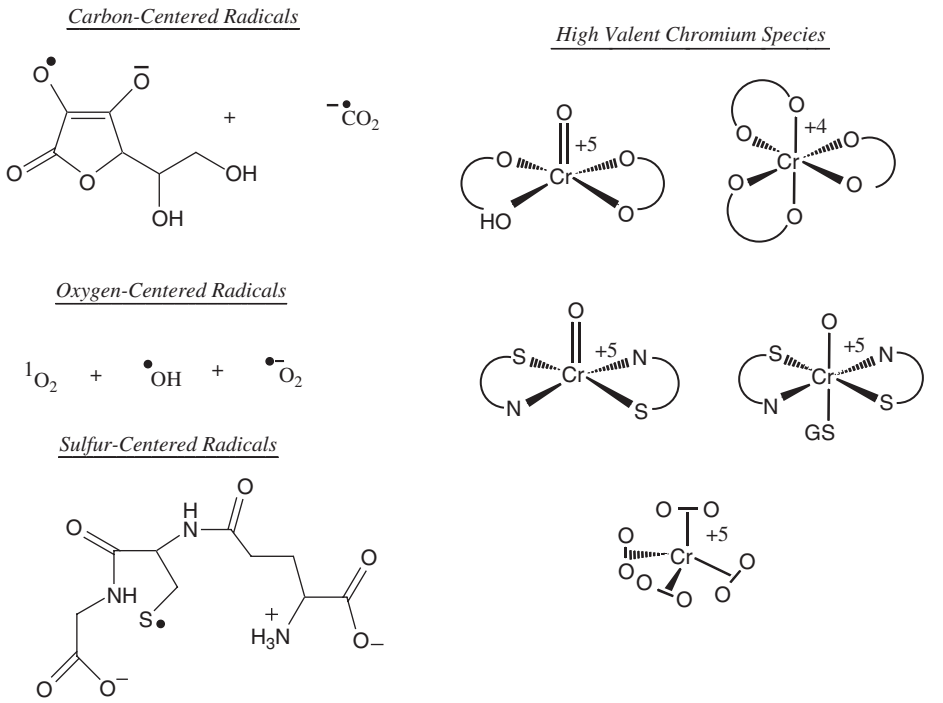


Figure 2 DNA reactive species formed during chromate reduction.

ascorbate is believed by most to be the principal reductant involved in chromate metabolism, although this finding should still be considered equivocal.

In the process of reduction, chromate generates a number of redox-active species including reactive oxygen species (ROS), carbon- and sulfur-centered radicals and high-valent chromium intermediate species, Cr(V) and Cr(IV) (Figure 2 [11–25]). Nearly, all of these species are capable of damaging DNA and yields of the different radical and metal species have been found to be dependent upon the type of reductant, the reductant/chromate ratio, pH, and oxygen concentration [11–25]. This wide assortment of potential DNA damaging agents has led to considerable discussion on the ultimate species responsible for DNA damage associated with chromate toxicity. Two different pathways have been proposed to account for DNA damage associated with chromate. These are the radical-mediated and the metal-mediated pathways.

2.1. Radical formation from chromate reduction

The radical-mediated pathway focuses primarily on the role that hydroxyl radical may play in the mechanism of DNA damage induced by chromate. Reaction of chromate with ascorbate and hydrogen peroxide [19] and chromate with glutathione and hydrogen peroxide [21,24] have all suggested hydroxyl radical

formation as the primary DNA damaging agent, Equations (1–2). The mechanism for hydroxyl radical formation is thought to involve a Fenton-like mechanism where initial one-electron reduction of Cr(VI), in this case by ascorbate (Asc), forms a Cr(V) species that can be re-oxidized by hydrogen peroxide to form the hydroxyl radical ($^{\bullet}OH$).

$$Cr(VI) + Asc \rightarrow Cr(V) + Asc^{\bullet -} \quad (1)$$

$$Cr(V) + HOOH \rightarrow Cr(VI) + {}^{\bullet}OH + OH^{-} \quad (2)$$

The generation of hydroxyl radical from peroxide during chromate reduction is compelling because of the well-defined DNA oxidation chemistry for Fenton metals such as iron and copper. However, the extremely low basal levels of peroxide in most living cells, 10^{-7} to 10^{-9} M [28,29], argues against this being a pathway of significant DNA damage. Alternatively, a number of other radicals have been observed to form during chromate reduction that are reductant-specific such as the carbon-based radicals from ascorbate [16,17,19] (the ascorbyl radical $Asc^{\bullet -}$ the formate radical $CO_2^{\bullet -}$) or sulfur-based radicals from glutathione [21,22,24] ($GSH^{\bullet}$), Figure 2.

To date there have been a number of studies supporting the formation and genotoxicity of reactive radical species *in vitro* and *in vivo*. However, the genotoxic contribution of metal-mediated pathway has been largely ignored relative to the radical-mediated pathway. O'Brien et al., recently addressed this issue in a comprehensive chromium carcinogenesis review [30].

2.2. High-valent chromium intermediate formation from chromate reduction

Along with the aforementioned radical species, intracellular reduction of chromate by endogenous reductants forms a number of transient high-valent complexes of chromium. The most easily observed of these are the Cr(V) complexes, Figure 2. Cr(V) is a d^1 metal complex that can be observed *in vitro* and *in vivo* using electron spin resonance spectroscopy, ESR or EPR. Studies have identified the formation of chromium(V) species from the exposure of chromium (VI) to human red blood cell suspensions [31], in cultured mammalian cells [10,32], in chick embryo red blood cells [33] and more recently in mouse organs and whole live mice [15,34,35]. Cr(V) is formed through a one-electron reduction such as that shown in Equation (1) with ascorbate. Cr(V) can also form through a comproportionation reaction between Cr(VI) and Cr(IV) to produce two Cr(V). Cr(V) is highly oxidizing with respect to Cr(VI) and many reactions with DNA have been suggested for this oxidation state.

$$Cr(VI) + Asc \rightarrow Cr(IV) + DHA \quad (3)$$

$$Cr(IV) + Asc \rightarrow Cr(III) + Asc^{\bullet -} \quad (4)$$

Cr(IV) is another transient high-valent chromium oxidation state that is formed through a two-electron reduction. An endogenous reductant such as ascorbate, when reacted with chromate, can form Cr(IV) and dehydroascorbate (DHA) through a two-electron transfer, Equation (3). Cr(IV) is a d^2 metal complex and as such is not readily observable by ESR. This spectroscopically "silent" valence state is less stable in comparison to Cr(V) and is quickly reduced to Cr(III). However, it should be noted that Cr(IV) is more oxidizing than either Cr(V) or Cr(VI) and may be the major oxidation state of chromium that causes DNA damage.

Irrespective of oxidation state, both Cr(IV) and Cr(V) are highly oxidizing and are candidates for the ultimate species responsible for much if not all of the DNA damage associated with chromate. In fact, many of the effects noted for chromate reduction *in vivo* and in cellular systems, and previously attributed to radical formation, have been observed to arise from the reaction of DNA with Cr(V) and Cr(IV).

3. Mechanisms of Chromate Genotoxicity

Chromium(VI) compounds are considered to be genotoxic based on the wide variety of DNA modifications that have been observed arising in bacterial systems, in mammalian cells and with *in vitro* DNA substrates following treatment with this metal. Chromate has been shown to produce DNA strand breaks [36–38], DNA–DNA crosslinks [39,40], DNA-protein crosslinks (DPCs) [41–43] and modified nucleic acid bases [44–46]. For the most part, these DNA modifications have not been structurally or mechanistically characterized but inferred by indirect methods such as alkaline elution and polymerase arrest. What is increasingly apparent in the study of chromate genotoxicity is that multiple and disparate mechanisms are necessary to account for the full suite of lesions that have been observed to form. The ultimate lesion(s) responsible for the DNA damage leading to the carcinogenic endpoint has been of particular debate and is the primary subject of this review.

The presence of the many types of DNA damage observed from Cr(VI) exposure has led the chromium community to postulate multiple pathways and mechanisms to account for this damage (as summarized in Figure 3). One of these pathways involves oxidative DNA damage with the formation of free radicals and the highly oxidizing chromium valence states, Cr(V) and Cr(IV), as the primary agents of this damage. The oxidative pathway is believed to give rise to the frank strand breaks, alkali-labile sites, and oxidized nucleic acid bases attributed to chromate exposure. The second pathway is a chromium-binding pathway that gives rise to Cr-DNA adducts and DPCs. The Cr-DNA adducts in this circumstance are considered to be conjugates of amino acids and endogenous reductants adducted to DNA through the base guanine and/or through the phosphate backbone.

Recent studies have begun to shed light on the structure and mechanisms of formation of DNA lesions that arise in both the oxidation and binding pathways from chromate exposure.

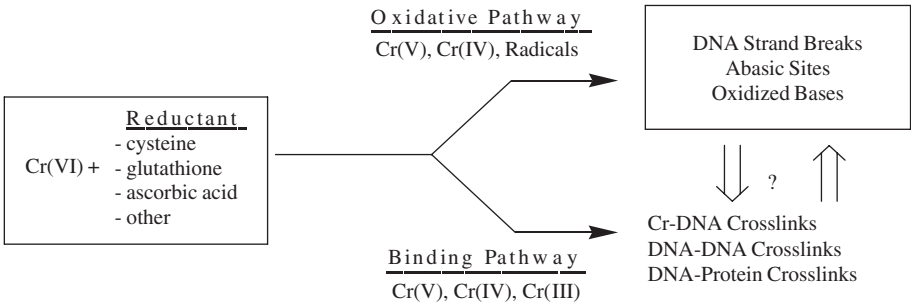


Figure 3 Putative mechanistic pathways for Cr-induced DNA damage.

3.1. DNA oxidation pathway

The ubiquity of oxidative stress, whether from normal metabolism or through the action of xenobiotics, is illustrated by the fact that even the simplest organisms have multiple cellular processes that have evolved to reduce and to mitigate its effects on biomolecules. Oxidative DNA damage as a result of Cr(VI) metabolism is believed to be one of the critical steps in chromium's carcinogenetic effects. DNA oxidation can occur either at the deoxyribose sugar or at one of the four nucleic acid bases. Depending upon the site of oxidation, different lesions with differing mutagenic and toxic endpoints can be formed.

3.1.1. Sugar oxidation and abasic site formation

Exposure to chromate has been shown to cause frank DNA strand breaks and abasic sites (manifested as piperidine-labile strand breaks) in bacterial and mammalian systems through a putative oxidative mechanism [36–38,47–50]. *In vitro* experiments with purified DNA substrates have demonstrated the ability of chromate to serve as a Fenton-like redox cycling substrate when in the presence of a reductant and hydrogen peroxide to induce strand breaks in a fashion similar to those observed with the "true" Fenton metals, copper and iron [51–53]. These findings led to the hydroxyl radical mechanism theory of DNA oxidation as the leading cause of strand breaks from chromate exposure. However there are a number of factors, most notably the extremely low levels of cellular hydrogen peroxide noted above, that suggests a direct metal-mediated mechanism of DNA oxidation as a rational alternative to the radical-mediated mechanism.

Frank strand breaks occur through oxidation at the deoxyribose sugar. The aliphatic nature of the deoxyribose moiety precludes an electron abstraction method but favors either a one-electron hydrogen atom abstraction mechanism, or a two-electron hydride abstraction mechanism. Hydrogen atom abstraction is thermodynamically favored at the tertiary hydrogens, 1′, 3′, 4′, over the secondary hydrogens, 2′S, 2′R, 5′R, 5′S, Figure 4, because of the enhanced stability of the resulting tertiary radicals over secondary radicals. However, in duplex DNA with a strong oxidizing agent, oxidation is controlled more by accessibility than by thermodynamics. The 4′ and one of the 5′ hydrogens are the most solvent exposed

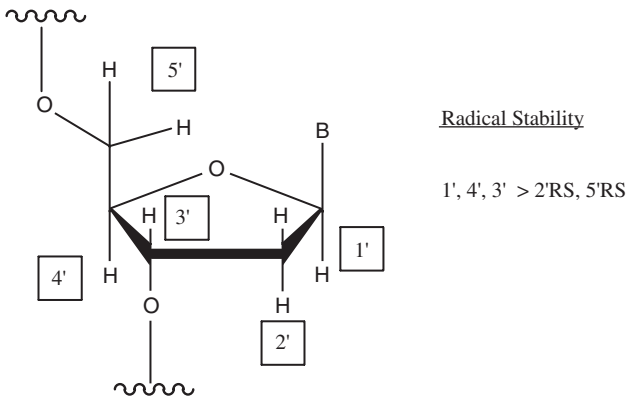


Figure 4 Deoxyribose structure showing hydrogen atoms available for abstraction.

in duplex DNA and are therefore more likely to be abstracted under these conditions [51].

Plasmid relaxation studies using chromate, a reductant and hydrogen peroxide as well as with synthetic Cr(V) complexes (designed to model this intermediate oxidation state formed during chromate reduction) were shown to nick and unwind supercoiled plasmid DNA [18,21,24,54]. In synthetic oligonucleotides, the formation of frank strand breaks during this process has shown little or no sequence specificity suggesting that the oxidation occurs at the deoxyribose moiety [55]. A model Cr(V) complex, Cr(V)-ehba, when reacted with nucleotides and synthetic oligonucleotides resulted in release of free DNA bases, the formation of 3′-phosphoglycolate termini and the formation of base propenals [48,49,55,56] (Figure 5). These products are hallmarks of 4′ hydrogen atom abstraction and are similar to that seen with hydroxyl radicals generated in a Fenton reaction [51–53] and with certain redox-active xenobiotics such as bleomycin [57]. Similarly, a 5′ hydrogen atom abstraction mechanism has been identified for a different Cr(V) complex, Cr(BT) or bis tris buffer complex of Cr(V) [47,58]. It is important to note that both 4′ and 5′ hydrogen atom abstraction mechanisms have been recognized as arising from a metal-mediated and not a radical-mediated pathway.

The hydrogen atom abstraction mechanisms proposed for DNA oxidation *via* high-valent chromium complexes accounts for two of the product arising from the oxidative pathway of Cr-induced DNA damage (Figure 3), strand breaks and abasic sites. However, these two products, while toxic, are nominally mutagenic and their contribution to chromates carcinogenicity is questionable.

3.1.2. Oxidized guanine lesions (8-oxoG, Sp and Gh)

7,8-dihydro-8-oxoguanine (8-oxoG) is the lesion that has historically been associated with DNA nucleobase oxidation. 8-oxoG has been shown to form from a variety of redox-active xenobiotics and endogenous metabolic processes. It has been estimated that 8-oxoG occurs at a frequency of ~10,000 bp per cell per day [59].

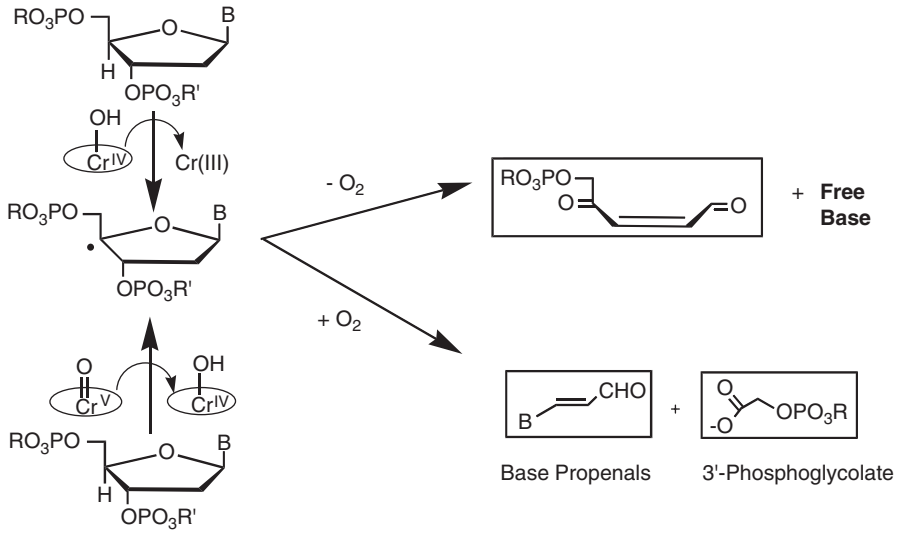


Figure 5 Simplified mechanism of C4′ sugar oxidation products.

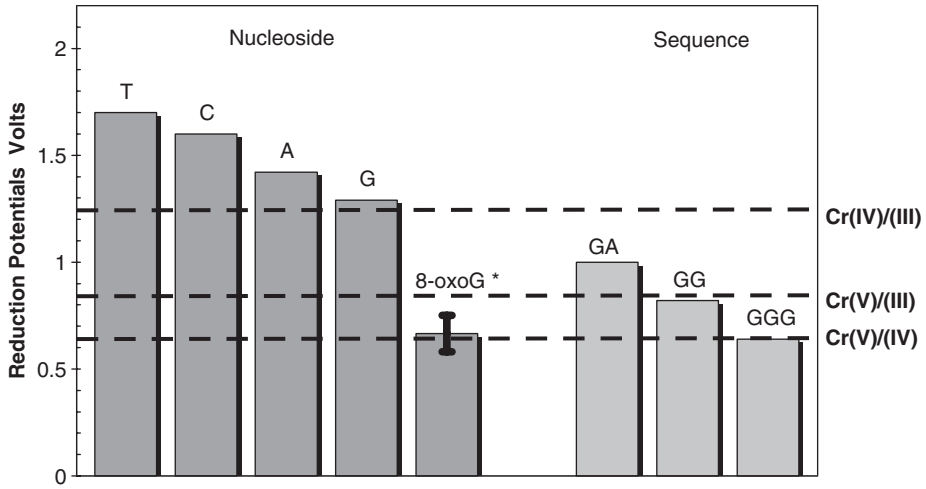


Figure 6 Reduction potentials of the four nucleobases, 8-oxoG, and guanine residues in DNA with consecutive runs. Dashed lines show the calculated reduction potentials of different high-valent oxidation states of chromium with respect to the nucleoside or DNA.

Because of this high frequency of occurrence, 8-oxoG has been implicated in the etiology of a large number of diseases and has been extensively used as a sensitive biomarker for oxidative damage to the cell [60–62]. The basis for the relatively high levels of cellular 8-oxoG formation is the enhanced sensitivity of the nucleobase guanine towards oxidation with respect to the other nucleic acid bases, Figure 6 [63]. This sensitivity of guanine towards oxidation is enhanced within duplex DNA

in consecutive runs of guanines at the 5′ of GG and GGG sequences [64]. In addition, the 8-oxoG lesion has a significantly lower reduction potential than the parent guanine, making it highly reactive towards further oxidation [65]. As illustrated in Figure 6, high-valent chromium species have reduction potentials adequate to oxidize guanine within duplex DNA as well as the 8-oxoG lesion as the free nucleoside [66]. This shows the thermodynamic basis for chromate's propensity to cause exclusive guanine nucleobase damage and form 8-oxoG and further oxidized guanine lesions.

The formation of 8-oxoG in DNA from chromate exposure has been shown in a variety of *in vitro*, cellular and *in vivo* systems. However, a number of inconsistencies exist in that not all chromate-treated systems have shown 8-oxoG formation. Irrespective of these inconsistencies, the mechanism associated with 8-oxoG formation by chromate has historically been tied to ROS production (pathways (a), and (c) in Figure 7) but recently, a number of other mechanisms have been postulated to account for the formation of this ubiquitous guanine lesion. Two such mechanisms are the electron abstraction mechanism, pathway (b) in Figure 7, and a metal-mediated oxo-atom transfer mechanism such as that shown in pathway (d) of Figure 7. Regardless of the mechanism, the final 8-oxoG product is the same for all pathways and discerning between these pathways to identify the ultimate species responsible for guanine oxidation is often difficult.

Recent studies have shown that 8-oxoG is considerably more prone towards oxidation than the parent base guanine, Figure 6. The lowered oxidation potential of this modified base allows the lesion to act as a thermodynamic sink for electron donation in redox reactions and potentially serve as a hot spot for further oxidation within DNA. A number of further oxidized lesions arising from 8-oxoG oxidation have been identified. A few of these; guanidinohydantoin, Gh, spiroiminodihydantoin, Sp, imidazolone, Iz, oxazolone, Oz, oxaluric acid, Oa, and parabanic acid, Pa, are shown in Figure 8 [44–46,67–72]. The first two of these species, Gh and Sp, have been observed to form specifically from the reaction of 8-oxoG with high-valent chromium and Sp has been detected in chromate-treated synthetic DNA substrates [44–46] and in repair-deficient *E. coli* [73].

In contrast to the hydrogen abstraction mechanism for deoxyribose sugars, oxidation of DNA bases occurs primarily through a one-electron abstraction mechanism. Electron abstraction produces a transient radical cation that is prone towards nucleophilic addition reactions that can result in the formation of a variety of different lesions depending on the respective base and nucleophile. Although water is a poor nucleophile it is kinetically favored due to its relative abundance and, dependent upon reaction conditions of pH and temperature, give rise to either Sp or Gh [70–72]. Mechanistically, the further oxidation of 8-oxoG through electron abstraction has been well studied by the Burrows group using the one-electron oxidant, Na_2IrCl_6, Figure 9 [70–72], and it is currently believed that chromate metabolism can mediate a similar one-electron mechanism.

As illustrated, the initial electron abstraction event at 8-oxoG leads to the formation of a radical cation intermediate that is prone towards nucleophilic attack from water. A secondary oxidation followed by a rearrangement that is pH

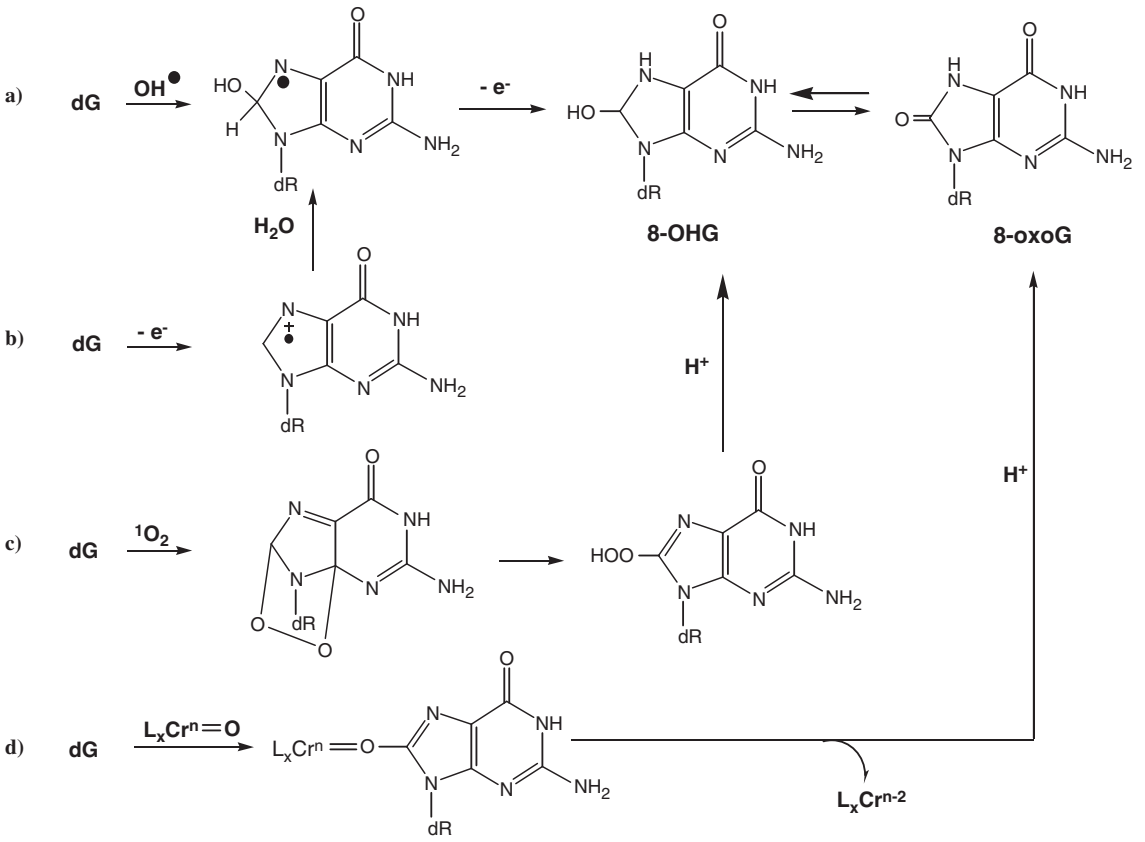


Figure 7 Multiple pathways of 8-oxoG formation by chromium or ROS.

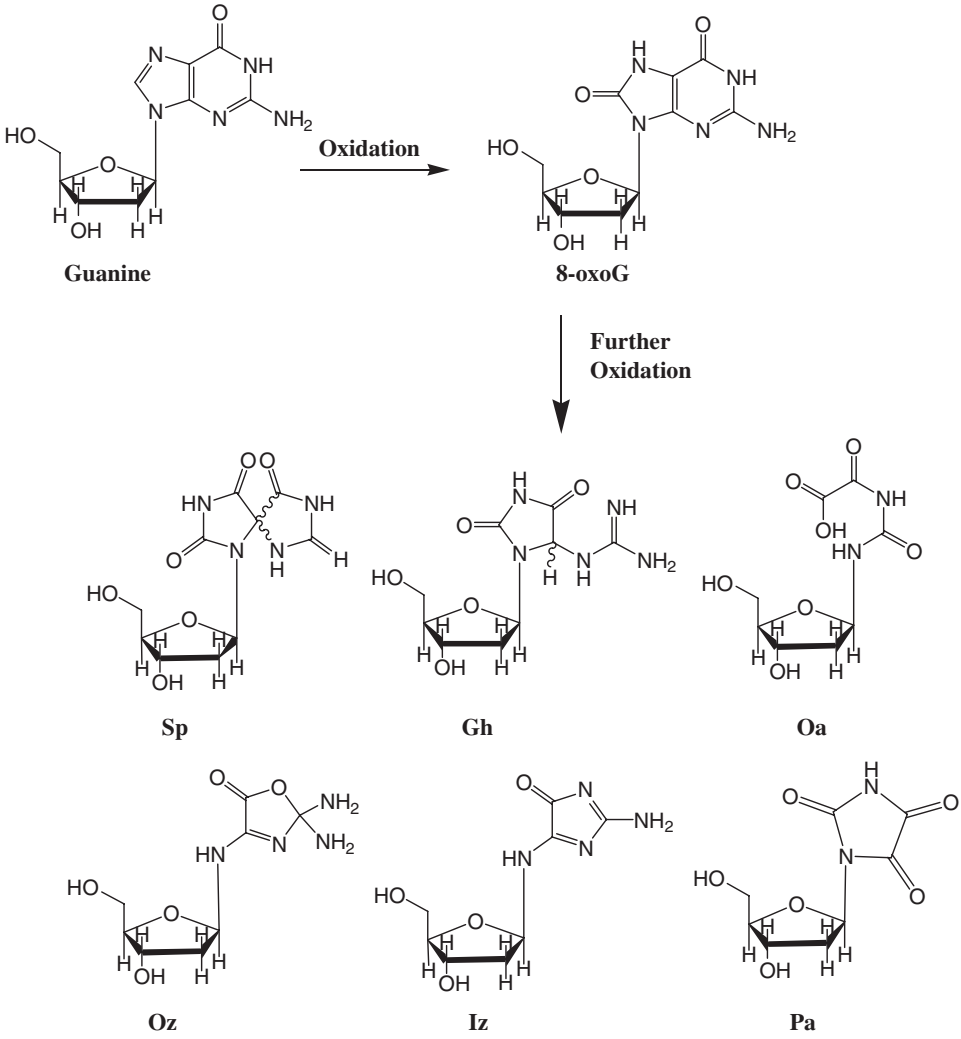


Figure 8 Structure of some further oxidized guanine lesions.

and temperature sensitive yields the final products, Sp and Gh. A second, discrete two-electron, mechanism for the formation of Sp from 8-oxoG has been proposed for high-valent chromium species such as Cr(V) that involves oxo-atom transfer. Isotope studies have shown that oxidations of 8-oxoG carried out in ^{18}O-water with the model Cr(V) compounds, Cr(V)-salen (*N*,*N*′-ethylene-bis-(salicylideneaminato)-oxochromium(V)), or Cr(V)-ehba (bis(2-ethyl-2-hydroxybutyrato) oxochromate(V)) showed enrichment for the natural ^{16}O isotope of oxygen at between 30 and 50%. This suggests that an oxo-atom transfer mechanism to form an intermediate epoxide at the C4–C5 alkene, and not nucleophilic attack of water at the C5, must be the initiating step [74] (Figure 10).

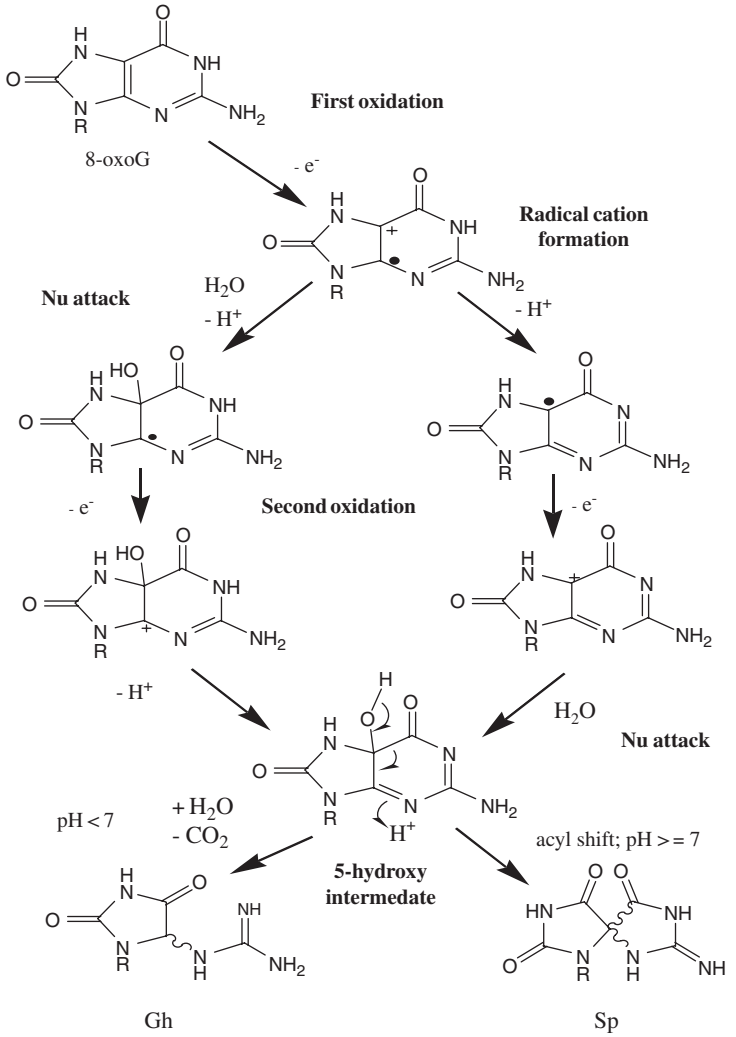


Figure 9 The mechanism for the formation of Gh and Sp from 8-oxoG oxidation.

While these hydantoin lesions have been identified from chromate exposure, their respective concentrations in eukaryotic cellular system and their contribution to the overall toxicity of this metal has yet to be determined. Unfortunately, *in vivo* identification and quantification of these lesions is refractory with respect to other DNA lesions such as 8-oxoG. However, where measured, chromate-exposed DNA from synthetic oligonucleotides and in a repair-deficient *E. coli* system have shown consistently elevated Sp concentrations relative to 8-oxoG [44,73]. Further studies are needed to allow definite conclusions but preliminary results suggest that further oxidized guanine lesions such as Sp and Gh may be more common than the "ubiquitous" 8-oxoG lesion. It should be noted that other mechanistic routes for the formation of further oxidized lesions exist and the preference for one over

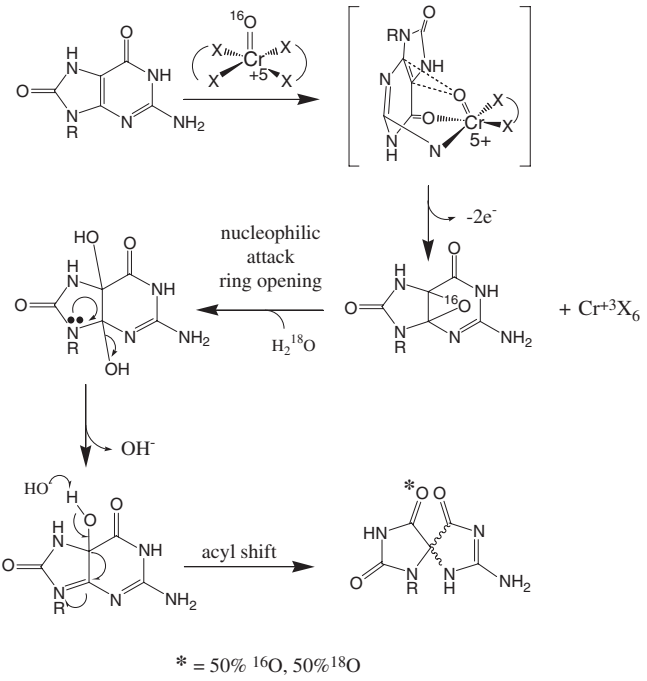


Figure 10 Two-electron mechanism for Sp formation by Cr(V).

another is governed by the oxidant and the conditions utilized. However, regardless of the mechanism of formation there is significant evidence suggesting that these lesions may be responsible for much of the cellular dysfunction observed under oxidative stress conditions.

In vitro studies on DNA base excision repair (BER) have shown that certain enzymes are able to recognize and remove the hydantoin lesions Oz, Oa, Sp, and Gh. Enzymes that have hydantoin recognition and cleaving ability include the bacterial BER glycosylases Endo III and Fpg [75–77], Nei (endonuclease VIII) [78], and eukaryotic BER enzymes yOGG1 and yOGG2 [75]. In addition the mammalian BER glycosylases NEIL1 and NEIL2 have shown a high affinity for recognition and cleavage of DNA containing Gh and Sp but almost no affinity for DNA containing 8-oxoG [79]. Given that eukaryotic BER enzymes are known to be highly substrate specific [80–82], the repair of lesions indirectly represents their existence and significance in cellular DNA. However, no studies on these lesions in a eukaryotic cellular system have been done and it remains unclear whether these lesions are *in vivo* cellular BER substrates.

The cellular relevance of further oxidized lesions has only begun to be established and their contribution to the overall genotoxicity of chromate is still poorly understood. Research is beginning to indicate that the formation of further oxidized guanine lesions have more serious implications to cellular toxicity and carcinogenesis than 8-oxoG. Studies with hydantoin lesions in *E. coli* have illustrated an order of magnitude greater mutation frequency for these lesions versus

8-oxoG [83–85]. As well, the 8-oxoG lesion generates exclusively G→T transversion mutations [83], while the hydantoin lesions generate both G→T and G→C transversion mutations [84,85]. In addition, Sp and Gh lesions have shown enhanced polymerase arrest relative to 8-oxoG [46,67,84–85]. The mutations commonly seen with these further oxidized lesions (G→T and G→C transversion mutations) are the primary mutations observed in the lung tumors of chromate-exposed workers and in shuttle vector replication assays in Cr(VI)-treated mammalian cells [86–89] providing indirect evidence that these lesions are likely to occur in cellular systems and are, at least partly, responsible for chromium carcinogenesis.

3.2. DNA-binding pathways

In order to account for the full suite of lesions produced following cellular exposure to chromate, a DNA-binding pathway has been proposed as well as the aforementioned oxidative pathway, Figure 3. Among the lesions that are thought to arise from this pathway are DPCs and ternary and binary adducts of DNA (comprised of chromium crosslinked DNA with amino acids and reductants such as glutathione and ascorbate). DPCs have long been considered a contributor to chromium toxicology and, along with ternary and binary DNA-reductant adducts, have been proposed as biomarkers of chromate exposure in cellular systems [90]. These crosslinks have been ascribed to the formation of complexes containing Cr(III) that serves to coordinate and bridge in a linear manner such as; DNA-Cr-protein or DNA-Cr-reductant [91]. Cr(III) forms stable six-coordinate complexes with many ligands and can associate with the phosphodiester backbone and possibly with DNA bases. Both coordinative and electrostatic interactions are possible and chromate metabolism has the potential to generate a host of genotoxic ternary adducts through this DNA-binding pathway. While many studies have indicated the propensity of chromium to produce Cr-DPCs, and ternary and binary adducts [92,93], a true understanding of this mechanism has been hindered by the inability to structurally identify these adducts. Recent studies surrounding the oxidative nature of chromate metabolism have revealed the possibility of an oxidant-mediated binding pathway that could form similar Cr-DPCs and ternary and binary adducts. Based on the known oxidative nature of chromate, this is an attractive hypothesis that may serve to unify the disparate mechanisms needed to account for nearly all the lesions associated with this metal.

3.2.1. Chromium-mediated binding pathway (ternary adducts and protein crosslinking)

Radiolabeled Cr^{51} chromate and C^{14}-labeled ascorbic acid, have been utilized to identify and quantify the formation of Cr-DNA adducts *in vitro*, in *E. coli* and in human cell cultures [20,90–95; for an excellent recent review, see reference 96]. It has been reported that intracellular metabolism of chromate leads to the formation of binary Cr(III)-DNA adducts and ternary Cr(III)-DNA adducts (Figure 11). Studies in mammalian cell culture have inferred that intracellular metabolism of

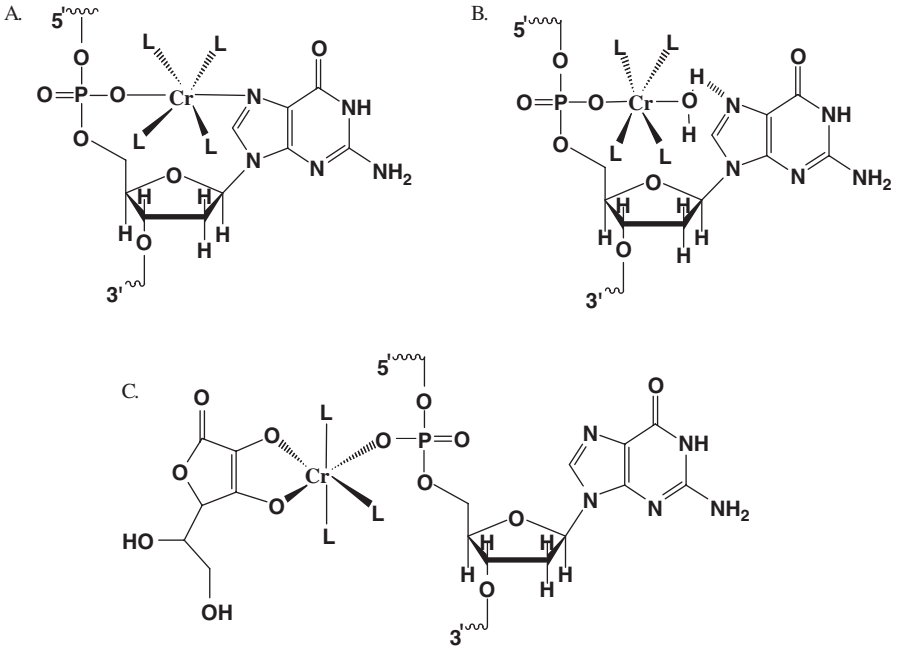


Figure 11 Proposed structures of Cr(III)-mediated DNA crosslinks.

chromate leads to the formation of DNA ternary adducts from Cr(III) coordinated with glutathione, cysteine, histidine, and ascorbate [90,91,93,94,97,98]. Yields of each species appear to be related to the intracellular concentration and reducing activity of the respective reductants [97]. Ascorbate metabolism of chromate has been reported to generate ternary Cr(III)-DNA adducts (structures A, B; Figure 11) and binary Cr(III)-DNA adducts (structure C; Figure 11) [87,88,91,94] *in vitro* with evidence to support ternary adducts as being far more mutagenic than the smaller and more transient binary Cr(III)-DNA adducts [96,99,100]. It should be noted that while both ternary and binary adducts have been inferred *in vitro*, the presence of Cr(III)-binary adducts in cellular systems have not been detected, most likely due to the high abundance of competitive intracellular ligands for Cr(III). In fact, Cr(III)s strong preference for coordination with phosphates and the observed ability of phosphate buffers and Mg ions to effectively inhibit Cr-DNA binding [20] has revealed the DNA backbone as the principal interacting site for Cr(III) complexes formed during ascorbate-mediated chromate reduction [94]. In addition, competing ligands such as phosphate buffer or EDTA have been shown to block ascorbate-mediated Cr-DNA interactions and reverse a vast majority of mutations [20] providing insight on the transient dynamics of Cr-DNA adduct interactions and their potential for genotoxicity.

Replication arrest studies have identified the potential for these Cr(III)-DNA adducts to be genotoxic by increasing nucleotide misincorporation and replication arrest [101–103]. This polymerase arrest was preferentially observed at DNA sites one base upstream from a guanine residue [101]. In addition, single base pair

mutations have shown a guanine-centered preference with predominately G/C → A/T transversion mutations [20,99,100]. While a number of studies provide evidence for a structural preference for the formation of Cr(III)-DNA adducts at guanine, the structure and site of the interaction(s) is still unknown. Studies on ascorbate-mediated Cr(III)-DNA binding to short oligonucleotides revealed a lack of base specificity [94]. In addition, nucleotide level mapping (exonuclease sequencing gel digestion) of Cr(III)-mediated cross-links of glutathione, histidine, and cysteine showed a lack of base specificity but subsequent mutations were found to be predominately single base substitutions at G:C base pairs [100]. While it is well understood that the phosphate backbone plays a vital role in chromium adduct formation, interactions between chromium and chromium bound ligands with base specificity requires more structural investigation.

A model for Cr(III) binding to guanine is the well-defined DNA coordination chemistry of the anti-tumor drug cis-platinum. Based on this model, it has been proposed that N7 of guanine acts in direct coordination in the formation of intrastrand crosslinks and ternary adducts [104]. However, cis-platinum, has unique coordination geometry, acid–base properties, and ligand lability that allows this metal to bind to the purine bases of DNA, and no structural studies have identified direct chromium binding to purine bases. The fact that the lone electron pair at the N7 is delocalized into the purine ring, making it a poor ligand for the binding of most metals, is in agreement with many experimental studies showing the transient nature of the majority of Cr(III) adducts with EDTA and phosphate [20,94]. It is clear that further investigation of Cr(III) adducts is needed especially in regard to the structural identification of these adducts. While transient adducts *in vitro* do not correlate well with toxicity in a cellular system, they have displayed an ability to cause genotoxic and mutagenic effects and the formation of these adducts should not be disregarded.

3.2.2. Oxidation-mediated binding

Direct coordination of chromium to DNA through guanine is not a compelling mechanism for formation of DNA adducts for the reasons described above. Recently, an alternative hypothesis for the mechanistic formation of crosslinks and DNA adduction has been proposed arising through an oxidative mechanism. Oxidation of DNA containing an 8-oxoG moiety by the one-electron oxidant, Ir(IV), in the presence of spermine was observed to form a covalent crosslink [105]. The Burrow's group has also shown that oxidation of 8-oxoG with Ir(IV) can form a specific lysine conjugated DPC with the BER protein, MutY [106]. This mechanism is compelling with regard to chromium adducts in that the mechanism is fundamentally the same as that which forms the Sp and Gh lesions described in Section 3.1.2. This mechanism proposes that oxidation of 8-oxoG generates a radical cation intermediate at the C5 of 8-oxoG which is prone towards nucleophilic attack. In the formation of Sp and Gh the nucleophile is water which, while kinetically favored with a concentration of ~55 M, is a relatively poor nucleophile with respect to the amino, sulfhydryl and carboxylato functional groups of the amino acids, glutathione, and ascorbate that are known to conjugate with

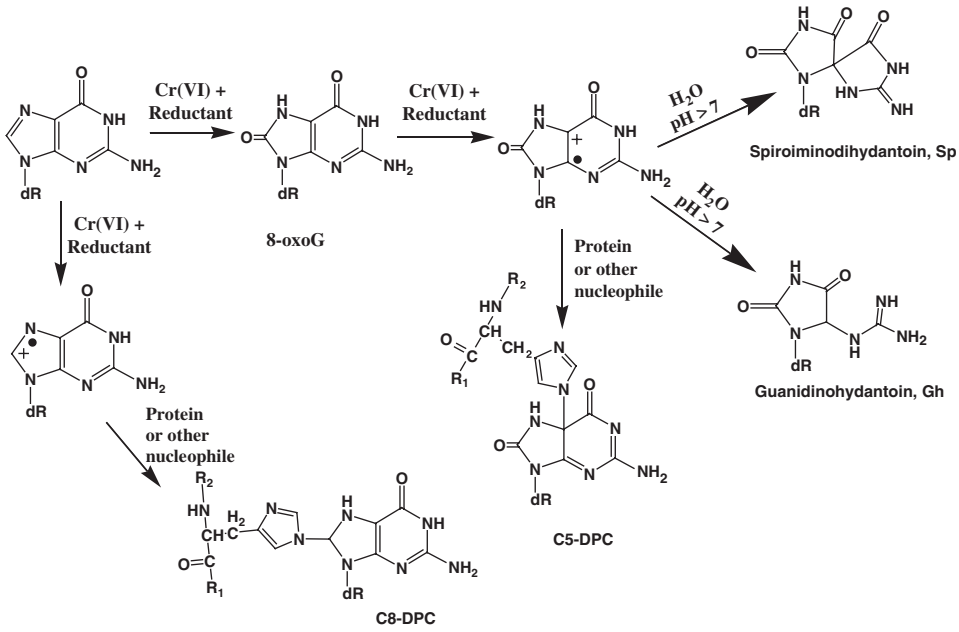


Figure 12 Proposed mechanism to account for chromate-induced DNA lesions arising from both the oxidative and binding pathways.

DNA following chromate treatment. A putative mechanism for the formation of an oxidative crosslink is shown in Figure 12. The final product of this mechanism is a C5-DNA-protein-crosslink (C5-DPC), which in this case is shown bound to a histidine side chain of a protein. The formation of this general type of DPC through an oxidative chromate-induced mechanism has been shown using the amino acid lysine and an 8-oxoG nucleoside as well as *via* oxidative crosslinking of an 8-oxoG containing oligonucleotide to the hOGG1 BER enzyme. It should be emphasized that the structure of the final conjugated product of this reaction in DNA is unknown. However, conjugation of lysine to the free 8-oxoG nucleoside following oxidation by chromate yields a spiro-like structure that has been tentatively identified as that shown in Figure 13.

A secondary mechanism for the formation of DPCs and DNA-reductant adducts has recently been suggested using photochemical reactions with guanine itself and not 8-oxoG [107,108]. This mechanism is shown in the lower part of Figure 12. A similar radical cation is generated with guanine as with 8-oxoG but at the C8 position. This leaves the final product as a C8-linked DPC (C8-DPC) as shown but one could easily envision conjugation with amino acids and reductants of chromate such as glutathione and ascorbate. Rearrangement of the C8-DPC has been shown to give rise to a number of final structures although it must be noted that none of these have yet been shown to arise from reaction of chromate with DNA.

Clearly, a potential exists for cellular DPC formation mediated by chromium oxidation due to the propensity of chromate metabolism to generate guanine radical

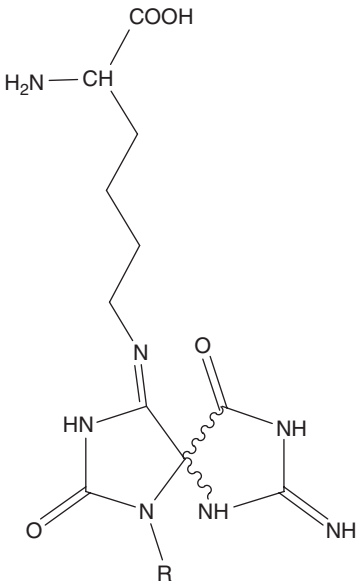


Figure 13 Proposed lysine conjugated spiro product derived from chromium oxidation of the 8-oxoG nucleoside in the presence of lysine.

cations, 8-oxoG, and a variety of further oxidized lesions. While initially driven by thermodynamics, the oxidative pathway appears to have a strong kinetic factor, especially in a cellular matrix where many other nucleophiles may compete with water for addition to the guanine or 8-oxoG radical cation intermediates (including known reductants of Cr(VI) such as glutathione and ascorbate as well as amine and sulfhydryl-containing amino acid side chains of proteins). In addition, the high abundance of proximal amino acid chains in proteins interacting with DNA such as histones, transcription factors, repair enzymes, and polymerases could provide a kinetic advantage to DPC formation in cellular systems. However, intracellular identification of these lesions is still needed to clearly define the biological relevance of DPC formation from an oxidative mechanism.

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CHAPTER 2

Drug-Induced Hepatotoxicity: Learning from Recent Cases of Drug Attrition

Axel Pähler* *and* Christoph Funk

Contents

1. Introduction

1.1. Causes of drug attrition

The development of new medicines that offer benefit for the treatment of unmet medical needs is a process that takes ten or more years from the early stages of target identification until the availability and selection of compounds that are finally developed, marketed and offered to patients after a positive regulatory approval. Major causes for removing certain chemical structures from further clinical

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evaluation relate to (pre)clinical toxicity (animal toxicity approximately 20%), lack of clinical efficacy (approximately 25%) and commercial considerations (approximately 20%) [1]. Unmanageable pharmacokinetics and metabolism in man [2] was a major reason for discontinuation of new drugs in early clinical trials one to two decades ago. Our understanding of fundamental aspects in drug metabolism and disposition as well as the availability of predictive *in vitro* assays and *in silico* extrapolations to the *in vivo* situation have considerably contributed to reduce drug attrition due to poor pharmacokinetics in man. Also, the availability of *in vitro* and *in vivo* methods to address certain aspects of drug safety early in preclinical development have helped to reduce drug attrition, e.g., in the areas of drug–drug interactions, cardiovascular safety or mutagenicity and carcinogenicity. One of the major reasons for potential drug failure in the recent years are adverse events in man with some toxicities appearing only once a drug has reached the market. Such cases of late stage, post-marketing attrition are of particular concern to patients and the pharmaceutical industry as they are unpredictable by conventional preclinical or early clinical testing strategies.

1.2. Clinical attrition due to hepatotoxicity

Adverse drug reactions (ADRs) are classified as pharmacology-related (type A) or as type B, C and D toxicities, mostly not related to a pharmacological effect [3,4]. For pharmacology-related mechanisms of toxicity, the effect may be either related to the primary pharmacology of the desired target (type A1) or to other secondary pharmacological effects (type A2, e.g., due to activity towards off-target receptors). In most cases, the pharmacological side effects are due to an exaggerated response in local tissues because of overdose, unfavorable tissue kinetics or the accumulation of active molecules (parent drug or metabolite). Usually these cases of toxicity involve the parent drug or metabolites with minor metabolic alterations or conjugation reactions during drug metabolism and are typically discovered significantly later than the pharmacological properties of the parent drug. Compounds falling into the categories of B, C and D types of toxicity, which typically show a low background frequency, share a common feature, namely the bioactivation of the parent drug molecule to toxic reactive, electrophilic metabolites capable of covalent binding to cellular macromolecules. Depending on the cellular target that is modified, the consequences may be acute [type C like for acetaminophen overdose and covalent binding of its *N*-acetyl-*p*-benzo-quinone imine (NAPQI) metabolite to cellular proteins], delayed (type D, e.g., for genotoxic agents that covalently modify tissue DNA *via* reactive metabolites like the anti-androgen cyproterone acetate) or due to immunological response (type B, so-called idiosyncratic ADRs). Whereas type D toxicities like genotoxicity and teratogenicity may also be related to secondary pharmacological or endocrinological effects, type B and C toxicities involve reactive metabolite formation in most incidences.

Serious adverse drug reactions are believed to be one of the leading causes of death in the United States. They were estimated to occur in over two million patients in 1994 with more than 100,000 fatal outcomes. Some studies estimate that hepatotoxicity is the major safety concern for discontinuation of clinical trials and the

post-marketing withdrawal of drugs [5]. More than 50% of the cases of liver failure in the U.S. are drug-induced with the majority attributed to acetaminophen overdose [6,7]. However, acetaminophen is well tolerated in the majority of patients at regular doses, leading to a favorable risk/benefit assessment. The discovery of late-stage serious adverse drug reactions including hepatotoxicity at a relatively high incidence at clinically efficacious doses has led to serious restrictions of the regular use ("black box warning") of several drugs, including bosentan, trovafloxacin, tolcapone, valproic acid, isoniazid, ketoconazole and felbamate among others. Recent cases of post-marketing withdrawal of drugs due to hepatotoxicity include tienilic acid, bromfenac, the thiazolidinedione (TZD) insulin sensitizer drug troglitazone (Rezulin) and the antidepressant nefazodone (Serzone) [8].

1.2.1. Troglitazone

The 2,4-thioazolidinedione-type insulin sensitizer troglitazone (Figure 1) was the first molecule of a new class of drugs used for the treatment of type 2 non-insulin-dependent diabetes mellitus [9]. During clinical development, 1.9% of the patients revealed a mild, reversible hepatotoxicity. After drug approval in 1997 between one and two million patients were treated with troglitazone until February 2000. Several cases of increased liver enzyme levels and several cases of fulminant hepatic failure were reported with increased levels of bilirubin and signs of hepatocellular necrosis and cholestasis [10–12]. Symptoms that occurred did not clearly correlate with the

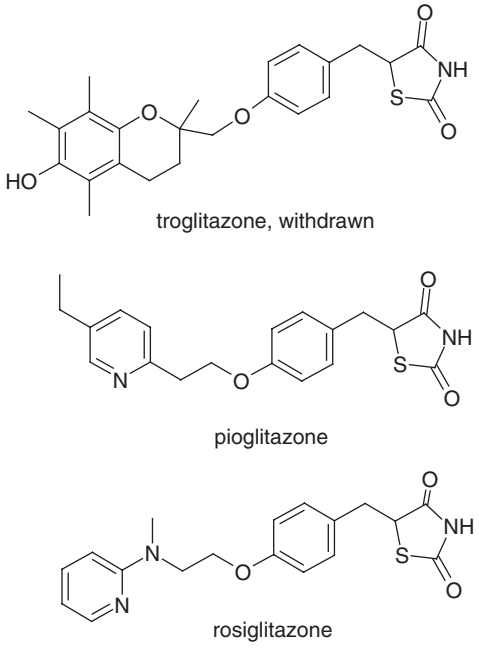


Figure 1 Chemical structures of the thiazolidinedione insulin sensitizer drugs troglitazone (withdrawn due to hepatotoxicity), pioglitazone and rosiglitazone.

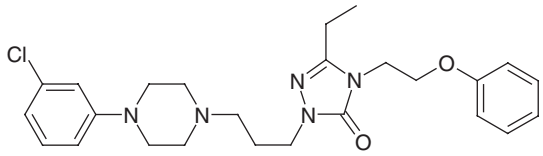
duration of treatment or dose. Frequent monitoring was insufficient to protect the patient population against adverse events. Troglitazone's unique therapeutic opportunity to control blood glucose in patients with diabetes versus the severe acute liver failures in approximately 1:20,000 patients complicated the risk/benefit assessment. Troglitazone was finally withdrawn from the market based on numerous reports of liver failure associated with its therapeutic use and the upcoming availability of alternative medications from the same chemical class (Figure 1).

1.2.2. Nefazodone

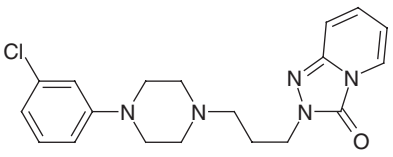
The antidepressant nefazodone (Figure 2) was introduced to the U.S. market in 1994. Despite the therapeutic benefits of the drug, several case reports of idiosyncratic liver failure were received by the U.S. Food and Drug Administration, including hepatobiliary dysfunction and cholestasis [13–17]. Since its market introduction nefazodone was associated with 55 cases of liver failure, including 20 deaths finally leading to the voluntary withdrawal of the drug by its manufacturer from the U.S. market in 2004. Previously, in a black-box warning in the drug's label, the rate of liver failure was estimated as about 1 case in 250,000 patients per year. This rate was about four-fold the background rate for liver failure and in contrast to no significant hepatotoxicity associated with other serotonin reuptake inhibitors. A liver biopsy from a patient with hepatic injury associated with nefazodone treatment revealed centrilobular necrosis consistent with a metabolism-related toxic liver injury.

1.2.3. Learning from troglitazone and nefazodone failures

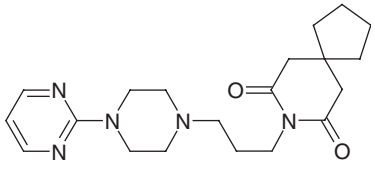
Possible mechanisms of troglitazone and lately nefazodone-induced liver toxicity have been deduced over the recent years and some controversy has emerged.



nefazodone, withdrawn



trazodone



buspirone

Figure 2 Chemical structures of the antidepressants nefazodone (withdrawn due to hepatotoxicity), trazodone and buspirone.

Metabolic and other factors involved in the idiosyncratic hepatotoxic effects produced by troglitazone have been debated [18,19]. Although circumstantial evidence suggests at least a partial contribution of metabolic drug bioactivation processes to their hepatotoxicity, other factors such as intrahepatic cholestasis as well as strong subject-dependent components seem to contribute further to the drug-induced fatal liver injuries.

Here we describe the at least plausible role of reactive metabolite formation leading to covalent protein binding in conjunction with other risk factors such as drug-induced intrahepatic cholestasis in the involvement of hepatotoxic events induced by both drugs. A preclinical strategy to address potential liabilities such as the propensity to generate excessive covalent binding *via* reactive metabolite formation or to induce intrahepatic cholestasis is presented. However, such a strategy would need to be challenged by comparison of the problematic prototype drugs troglitazone and nefazodone with their corresponding marketed analogs rosiglitazone, pioglitazone, buspirone and trazodone (the latter associated with less frequent hepatotoxic events as compared to nefazodone). These compounds have to be assessed in parallel to their "problematic" comparators in these preclinical models presented addressing potentially adverse drug properties. The accumulated evidence for the underlying mechanisms of hepatotoxicity induced by both drugs, the recent advances in technology related to molecular toxicology and the understanding of the biochemical processes involved can be a starting point to proactively develop predictive approaches for avoiding idiosyncratic liver injury. The outcome of this characterization will be discussed in the context of the marketed safe analogs and potential implications for an informed risk assessment will be provided.

2. Postulated Mechanisms of Hepatotoxicity

2.1. Metabolic drug activation and idiosyncratic liver injury

At present, no assay can predict the propensity of a new chemical entity to cause idiosyncratic reaction(s) or severe ADRs with respect to hepatic injury in the clinic. Idiosyncratic drug reactions are characterized by low frequency, are not predictable and do not occur in most patients at clinical doses. Circumstantial evidence indicates that reactive metabolites and subsequent covalent binding to cellular targets (hapten formation) are involved in the vast majority of cases (Figure 3) [20,21]. However, additional common risk factors seem to be necessary for drug-induced hepatotoxicity representing a so-called "danger signal" for the cell [22,23]. These include the potential of a new chemical entity to (i) induce hepatic oxidative stress, (ii) disrupt the mitochondrial membrane potential, (iii) interfere with hepatic active transport processes like the elimination of bile-acids leading to intra-hepatic cholestasis or, (iv) the induction of cellular necrosis or apoptosis leading to cell death. These risk factors might be attributed to an underlying disease or to a susceptible sub-population that is at particular risk of hepatotoxicity. Thus, idiosyncratic drug reactions are characterized by a strong

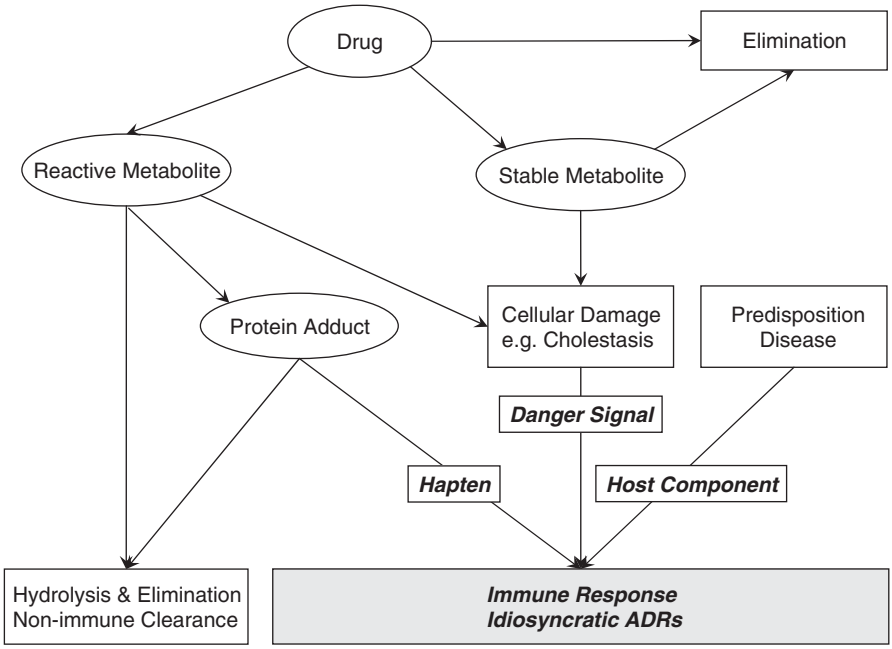


Figure 3 Involvement of reactive metabolites and haptenized proteins in metabolic idiosyncratic drug reactions.

host-dependent component. For almost all drugs associated with severe liver ADRs, bioactivation mechanisms have been proposed and/or reactive metabolites have been identified [24,25]. It is important to note that a compound that tests positive in the assays currently available for reactive metabolite assessment (covalent binding to microsomes, glutathione adduct formation or time-dependent inhibition) may not necessarily induce hepatic or idiosyncratic toxicity in humans. Short-lived electrophiles may be rapidly hydrolyzed and eliminated as stable products without reacting with critical target proteins. Also, non-immune clearance of protein adducts that are not critical for cell function and that do not trigger immune-response is another important protective mechanism of the cell. Probably covalent binding to cellular proteins remains asymptomatic and "silent" in the majority of cases. Furthermore, hepatic injury occurring in the clinic is often not seen in preclinical toxicity studies. Covalent binding of drugs to critical cellular macromolecules has been implicated in the toxicity of a number of drugs, although the molecular mechanism underlying the toxicity has not been clearly identified. Occasionally the drug itself is protein-reactive but in the majority of cases it is a reactive, electrophilic metabolite that binds to the cellular target (e.g., cytochrome P450 enzymes and other proteins). Once generated, electrophilic metabolites react with nucleophilic target sites and form covalent adducts. It has been generally recognized that there is a correlation between covalent protein adduct formation and certain forms of hepatic toxicity. Covalent adduct formation of reactive metabolites with liver proteins could lead to a number of downstream events that

may be involved in liver injury. These include (i) direct toxicity by binding to or inactivating a specific protein critical for cell survival, (ii) direct toxicity by binding to a large number of proteins resulting in disruption of cell homeostasis, (iii) covalent modification of proteins, leading to the formation of a neo-antigen and possibly to an immune response, and (iv) activation of a signaling cascade.

Chemical reactivity of electrophilic metabolites with cellular macromolecules follows well-understood mechanisms of chemistry and target different nucleophilic sites in proteins or DNA depending on the nature of the electrophile formed [26]. It is a widely accepted feature of certain functional groups to be susceptible to reactive metabolite formation mostly during phase I drug metabolism reactions. These so-called "structural alerts" include aromatic amines (prone to N-oxidation), *para*-hydroxy amines or amides (like in acetaminophen), thiophenes (tienilic acid), or simple functionalities like carboxylic acids (conjugated to reactive acyl glucuronides as for bromfenac) prone to reactive metabolite formation. Covalent alterations of cellular structures or redox cycling of reactive intermediates such as quinones have been shown to be involved in a variety of drug-related toxicities. Direct acting agents such as acetaminophen follow mostly classical dose–response relationships in their disruption of critical cellular functions. Type B toxicities do not necessarily follow classical dose–response curves although some evidence suggests at least a partial contribution of high doses to the risk associated with idiosyncratic toxicities [8,27]. Toxicities that are largely dependent on individual susceptibility are rare occurrences and hence are currently not amenable to prediction from animals or *in vitro* data. Numerous cases of post-marketing attrition especially for those involving hepatic toxicity have been linked to reactive metabolite formation. The underlying molecular mechanisms of drug bioactivation leading to covalent modifications of cellular macromolecules are well documented [24,25,28].

Many cases of drug-induced liver toxicity have been demonstrated in humans to involve covalent binding of reactive metabolites to proteins. This is not only true for intrinsically hepatotoxic drugs given at high dose (such as acetaminophen, methapyrilene) but also for many drugs causing idiosyncratic hepatic toxicity (e.g., halothane, dihydralazine, tienilic acid, bromfenac or felbamate). These considerations indicate that covalent binding to proteins is indeed an important biomarker reflecting the presence of potentially harmful reactive metabolites.

2.1.1. Bioactivation of troglitazone and other thiazolidinedione drugs

The mechanisms of troglitazone-induced hepatotoxicity are not fully understood and seem to be multifactorial [18], one of the potential factors being the formation of reactive metabolites after enzyme-mediated bioactivation. Clinical and post-marketing surveillance indicated that troglitazone-induced hepatotoxicity was not a characteristic of TZD drugs in general although isolated case reports of hepatotoxic events for rosiglitazone and pioglitazone exist [29,30]. Thus, not only the understanding of bioactivation processes common to all TZD drugs but also the characterization of troglitazone-specific risk factors related to drug metabolism seems of importance if one aims at identifying potentially harmful metabolism

properties of TZD drugs. Also, the particularly high therapeutic dose of troglitazone in comparison to rosiglitazone and pioglitazone needs to be judged in this context as potentially contributing risk factor to the drug's risk.

Major hepatic clearance of troglitazone is mediated by the formation of conjugated phase II glucuronide and sulfate metabolites, the latter metabolite representing the major drug-related material in rat liver after troglitazone treatment. In addition to phase II metabolism pathways, troglitazone has been shown to undergo bioactivation processes in rat and human liver microsomes leading to the formation of reactive intermediates capable of covalent binding to protein thiol groups. The major pathway for troglitazone metabolism is P450 3A4-mediated oxidation of the chromane moiety to a potentially harmful quinone metabolite, followed by reduction and sulfation [31,32]. Kassahun et al. described the formation of several reactive intermediates that were trapped as glutathione (GSH) adducts *in vitro* and in rat bile *in vivo* [33] involving bioactivation of either the TZD ring or the chromane moiety. Others reported the detection of troglitazone-derived GSH adducts in human hepatocytes [34] as well as in rats *in vivo* [31,35].

The bioactivation potentials of the three TZD drugs troglitazone, pioglitazone and rosiglitazone were recently investigated by our group to determine whether the safe analogs pioglitazone and rosiglitazone are also prone to reactive metabolite formation *via* TZD-ring-opening processes [36]. *In vitro* studies using either non-labeled drugs or TZDs with stable isotopes 2-^{15}N-3,4,5-$^{13}C_3$ incorporated in the unique structural motif required for the drugs' pharmacological activity (Figure 4A) were conducted for the detection of TZD-derived GSH conjugates. The use of different isotopes combined with liquid chromatography and mass spectrometric techniques facilitated the detection and characterization of the drug GSH conjugates due to the shift in molecular weight of the labeled and unlabeled GSH conjugates. This approach employing stable isotope labeling strategies gave valuable support for the identification and structural elucidation of TZD-related bioactivation pathways.

The predominant GSH adduct of troglitazone **T1** dominated the survey scan chromatograms obtained in positive ion mode for the generic detection of glutathione adducts (Figure 5C) in contrast to the less abundant GSH adducts derived from TZD ring scission. **T1** that is formed in rat and human liver microsomes showed the conservation of all four labeled positions of the TZD-moiety. Product ion spectra of the major troglitazone-derived glutathione adducts in negative ion mode, revealed a 4 amu difference between the unlabeled and labeled adduct of the activated troglitazone quinone–methide adduct (Figure 6) consistent with the structure reported by others (Figure 4B) [33].

Troglitazone further undergoes P450-mediated S-oxidation of the TZD moiety that is suggested to lead to an unstable TZD-sulfoxide which undergoes spontaneous ring opening to form a reactive sulfenic acid-α-keto-isocyanate [33] (Figure 4A). The minor adduct **T2** also reveals a 4 amu difference to its labeled analog– but involved TZD ring-scission affording a disulfide-type glutathione adduct. **T4** exhibited a 3 amu difference in molecular weight between the non-labeled and labeled derivatives due to a loss of $^{13}CO_2$ from the TZD ring (Figure 7). Subsequent oxidative deamination resulted in the generation of carboxylic acid-type mixed-disulfide GSH

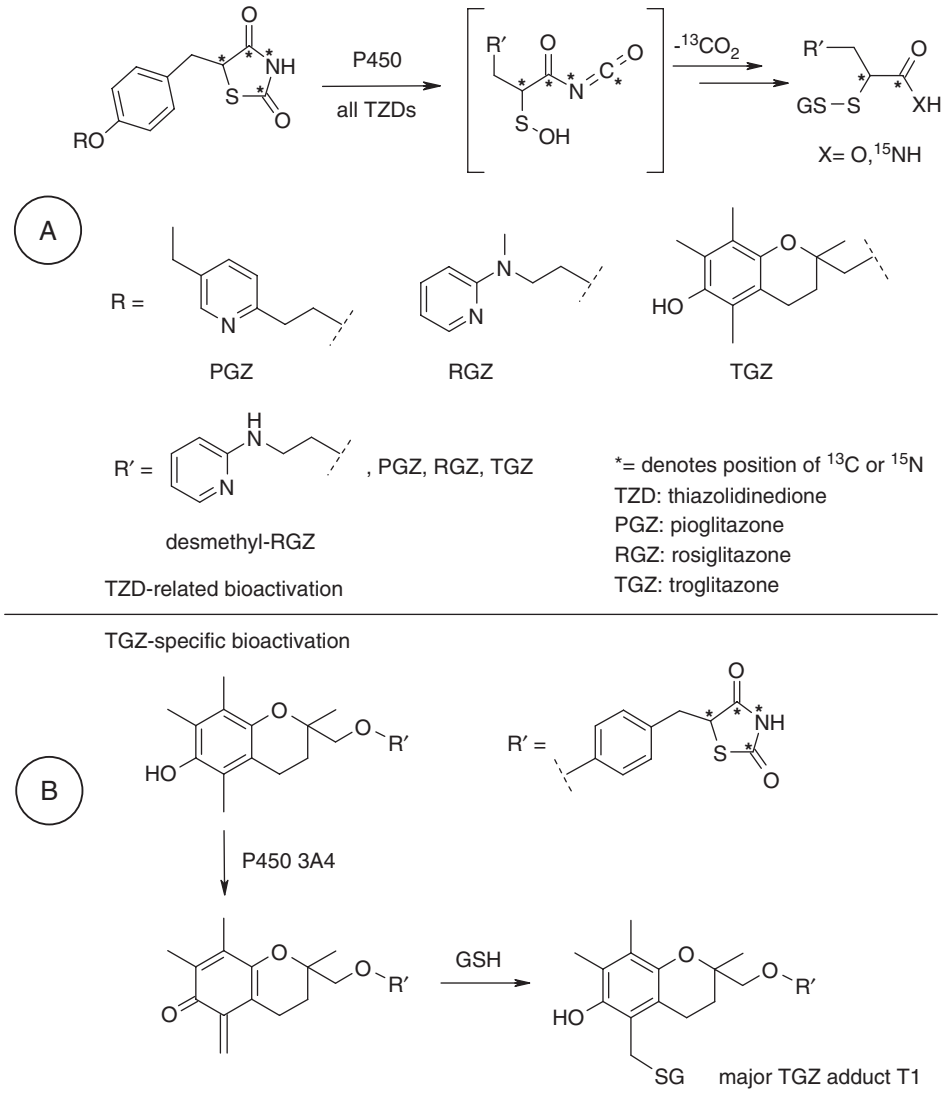


Figure 4 Thiazolidinedione drugs and their stable labeled analogs. Asterisks denote the labeled positions of the corresponding 2-^{15}N-3,4,5-$^{13}C_3$-labeled analogs. (A) Sequential metabolism of TZD drugs *via* TZD ring-scission involving loss of labeled positions during bioactivation and glutathione adduct formation. (B) Troglitazone-specific bioactivation involving quinone–methide formation and subsequent formation of a glutathione sulfhydryl adduct.

conjugates, revealing additional loss of the ^{15}N-position. The resulting GSH conjugate **T3** showed a 2 amu difference in molecular weight to its corresponding labeled analog.

Pioglitazone undergoes predominantly hydroxylation of the ethyl side chain by P450 3A4 and 2C8 followed by sulfation, glucuronidation or taurine conjugation [37,38]. Similar to troglitazone, TZD-ring-opening biotransformation reactions

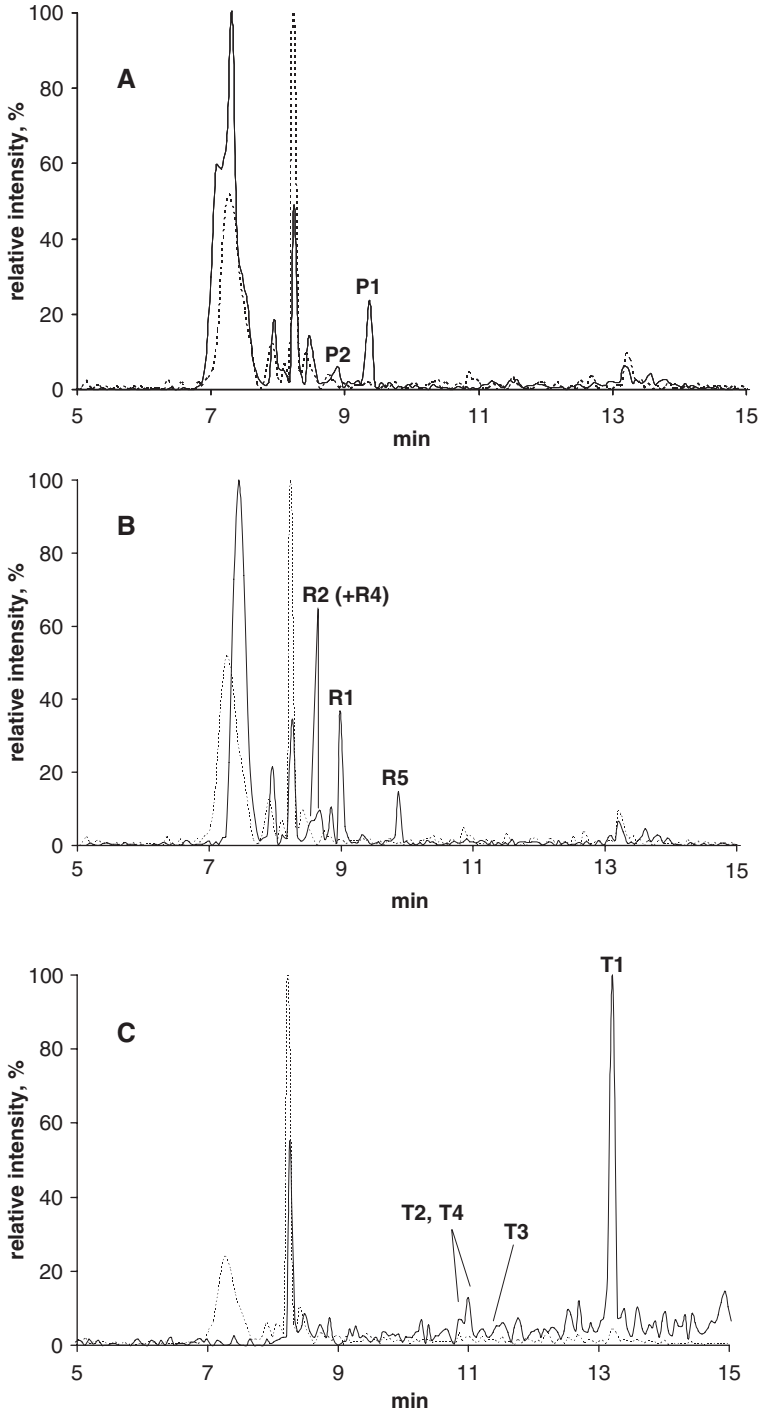


Figure 5 Extracted ion chromatograms (XIC) from rat liver microsomal incubations of TZD drugs in the presence of glutathione (solid line) and blank incubations performed in the absence of substrate (dotted line). (A) Pioglitazone (CNL scan 129 amu, positive ion mode, XIC *m/z* 600–700). (B) Rosiglitazone (CNL scan 129 amu, positive ion mode, XIC *m/z* 600–700). (C) Troglitazone (CNL scan 129 amu, positive ion mode, XIC *m/z* 700–800).

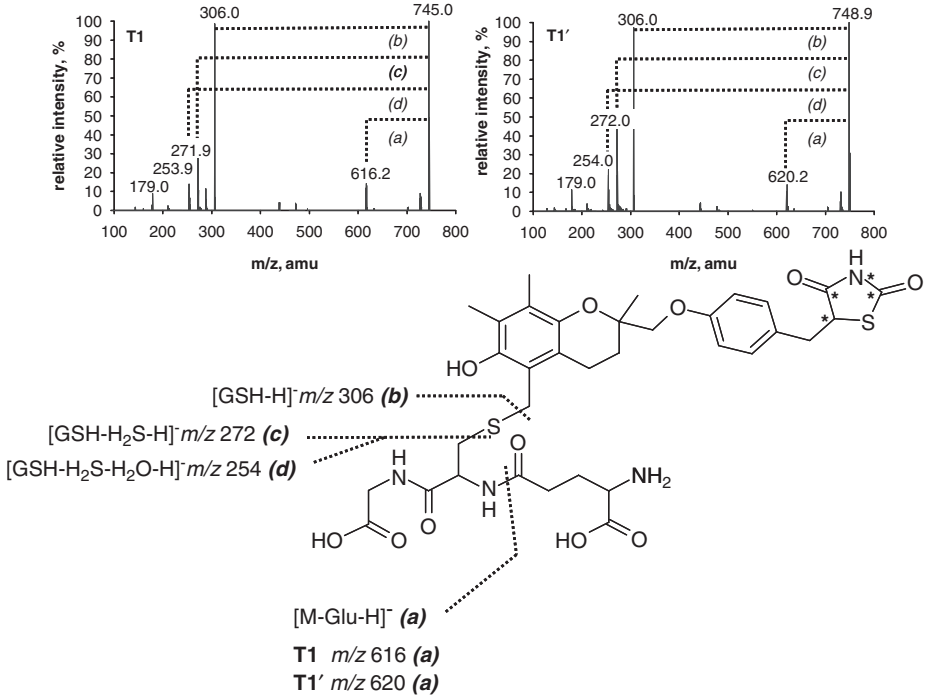


Figure 6 (Top) Enhanced product ion spectra (negative ion mode) of the major troglitazone GSH conjugate detected in human liver (T1, $[M-H]^-$ at *m/z* 745, non-labeled) and T1′ ($[M-H]^-$ at *m/z* 749, labeled). (Bottom) Structure of the quinine–methide derived GSH adduct with all four labeled position conserved. Labeled positions are indicated with asterisks.

have been described for pioglitazone in dog liver microsomes [39]. Baughman et al. [40] reported on the formation of pioglitazone-derived TZD-ring-opened GSH adducts similar to the mixed-disulfide amide and carboxylic acid derivatives described for troglitazone. Rosiglitazone is metabolized predominantly *via* P450 mediated N-demethylation and hydroxylation followed by glucuronidation [41]. A rosiglitazone-derived GSH adduct in human liver microsomes fortified with GSH was reported by Soglia et al. [35] using LC-ESI/MS/MS and a targeted multiple reaction monitoring method. Besides troglitazone, rosiglitazone and pioglitazone also are prone to form reactive intermediates at higher substrate concentrations, suggesting that covalent binding to liver proteins may also be expected for these two compounds (Figure 5A and B). In our comparative study, mixed-disulfide type GSH conjugates were detected for rosiglitazone and pioglitazone similar to troglitazone. The loss of carbon dioxide as a result of bioactivation on the TZD moiety during GSH adduct formation resulted in a characteristic change of the isotopic difference between the non-labeled and labeled analogs of rosiglitazone and pioglitazone (Figure 4A). The mixed-disulfide GSH conjugates of the amide type derived from pioglitazone (**P2** Figure 8) and rosiglitazone (**R2** Figure 9) exhibited a 3 amu difference in molecular weight

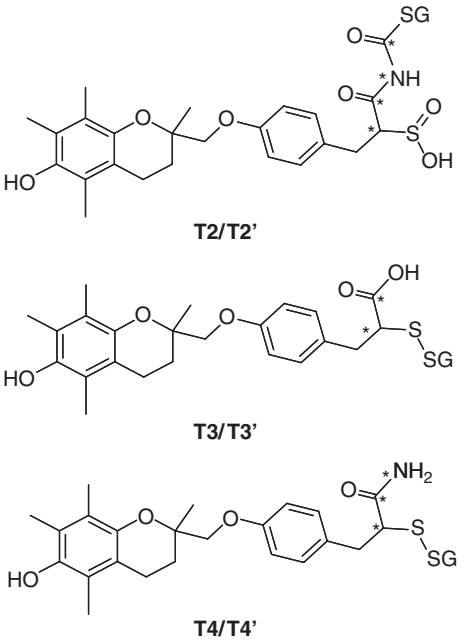


Figure 7 Structures proposed by Kassahun et al. [33] for T2 ($[M-H]^-$ at *m/z* 779), T3 ($[M-H]^-$ at *m/z* 720) and T4 ($[M-H]^-$ at *m/z* 719). Experiments performed with the labeled TGZ derivative show a 4 amu increase in mass for T2′ ($[M-H]^-$ at *m/z* 783), 2 amu for T3′ ($[M-H]^-$ at *m/z* 722) and 3 amu for T4′ ($[M-H]^-$ at *m/z* 722), in agreement with the proposed structures. Stable isotope-labeled atoms are indicated with asterisks.

between the non-labeled and labeled derivatives due to a loss of $^{13}CO_2$ from the TZD ring. Similar to troglitazone, subsequent oxidative deamination resulted in the generation of carboxylic acid-type mixed-disulfide GSH conjugates **P1** and **R1** with a 2 amu difference in molecular weight to their corresponding labeled analogs.

TZD-dependent bioactivation occurs for all TZD drugs in rat and human liver microsomes. Studies from our group and others have revealed that rosiglitazone and pioglitazone are also prone to form reactive intermediates at higher substrate concentrations, suggesting that covalent binding to liver proteins may also be expected for these two compounds. In human liver microsomes at low drug concentrations the formation of GSH adducts was evident for troglitazone but almost not detectable for rosiglitazone and pioglitazone. For troglitzazone, the non-TZD-dependent GSH adduct **T1** was the predominant product detected. TZD ring scission represents a minor metabolic pathway for troglitazone and pioglitazone [33,39]. This observation suggests that for troglitazone bioactivation *via* TZD ring scission does not contribute significantly to GSH adduct formation. Although the relative contribution of less abundant reactive intermediates to hepatotoxic events remains unknown these findings suggest that the observed hepatotoxic potential of troglitazone might be associated with the formation of reactive intermediates not related to the TZD-moiety.

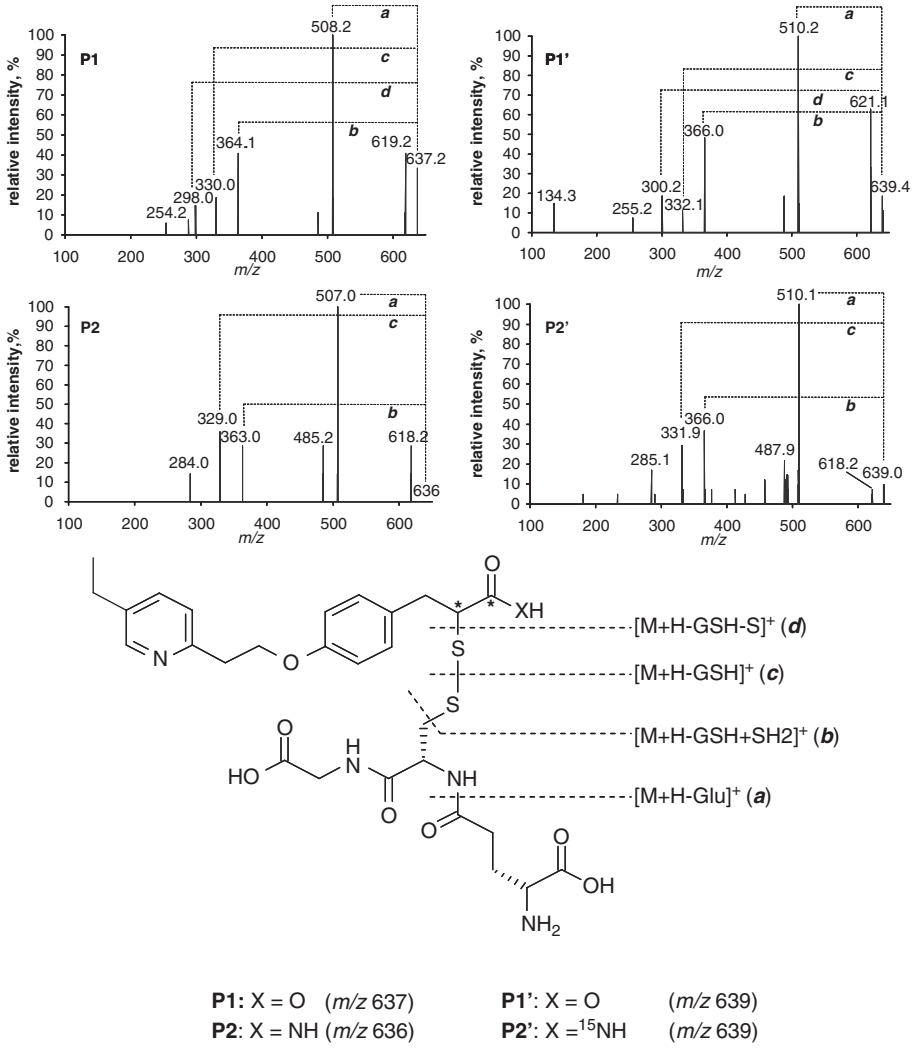


Figure 8 (Top) Enhanced product ion spectra (positive ion mode, collision energy 35) of the pioglitazone GSH conjugates detected in liver microsomes. P1 ($[M+H]^+$ at *m/z* 637, non-labeled), P1′ ($[M+H]^+$ at *m/z* 639, labeled), P2 ($[M+H]^+$ at *m/z* 636, non-labeled) and P2′ ($[M+H]^+$ at *m/z* 639, labeled). (Bottom) Structures proposed for these conjugates and their main characteristic MS/MS fragments. Labeled positions are indicated with asterisks. Letters in bold italic on the structure refer to the corresponding fragmentation in the MS/MS spectra.

2.1.2. Bioactivation of nefazodone and its safe analogs

Therapy with the antidepressant nefazodone was associated with several fatal cases of drug-induced liver failure leading to the withdrawal of the drug from the U.S. market in 2004. Symptoms and characteristics of nefazodone-induced hepatotoxicity were consistent with a metabolism-related toxic liver injury [42].

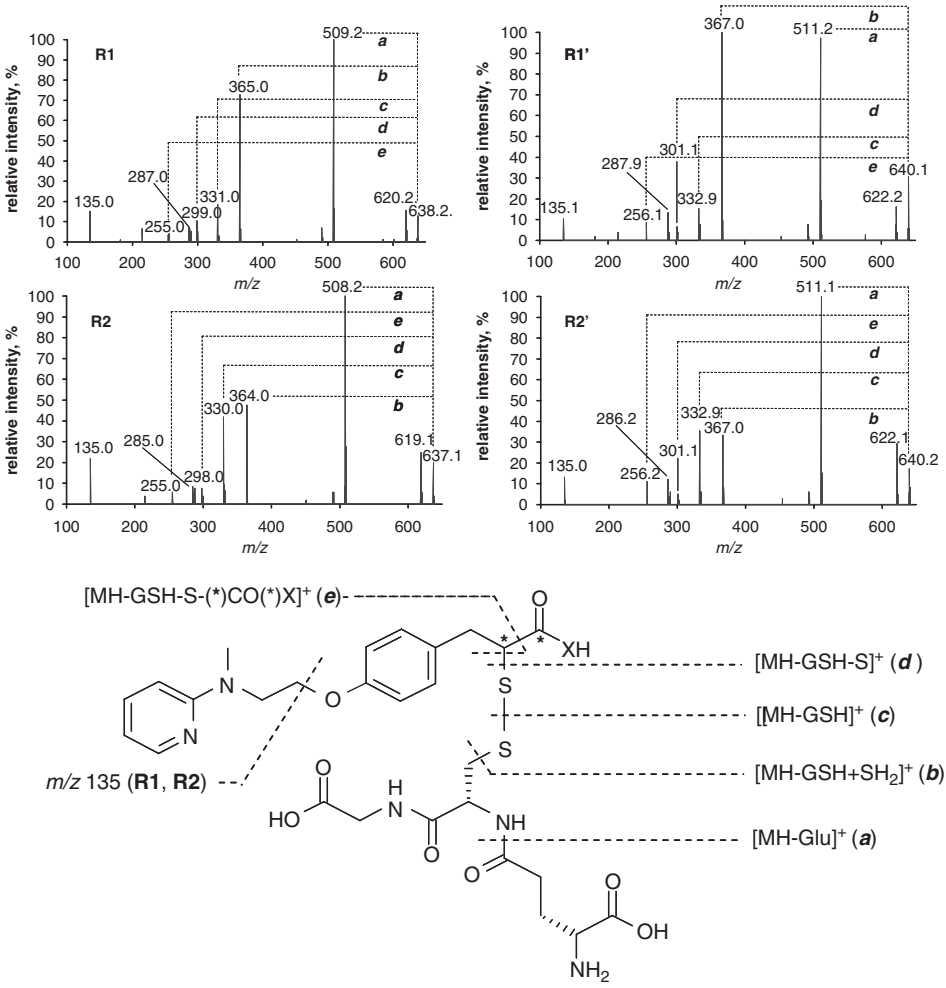


Figure 9 (Top) Enhanced product ion spectra (positive ion mode, collision energy 35) of the GSH conjugates of rosiglitazone. R1 ($[M+H]^+$ at *m/z* 638, non-labeled), R1′ ($[M+H]^+$ at *m/z* 640, labeled), R2 ($[M+H]^+$ at *m/z* 637, non-labeled) and R2′ ($[M+H]^+$ at *m/z* 640, labeled). (Bottom) Structures proposed for these conjugates and their main characteristic MS/MS fragments. Labeled positions are indicated with asterisks. Letters in bold italic on the structure refer to the corresponding fragmentation in the MS/MS spectra.

The metabolism of nefazodone in human hepatic microsomes has been studied extensively. The major route of hepatic nefazodone clearance is metabolic transformation by cytochrome P450 3A4 [43–45]. Nefazodone metabolism leads to numerous polar biotransformation products that recently have been studied in a novel mass spectrometric approach employing high resolution accurate mass

spectrometry [46]. Besides primary oxidation reactions, nefazodone also undergoes N-dealkylation and gives rise to chlorophenyl-piperazine (m-CPP; Figure 10A). Regarding potential bioactivation processes leading to electrophilic metabolites capable of covalently modifying hepatic proteins, those primary and secondary phase I metabolites related to the aromatic hydroxylation occurring in *para*-position to the piperazine nitrogen are of particular interest. These metabolites carry a particular structural alert and may be subject to further oxidation processes yielding potentially protein-reactive quinone-iminium species. *Para*-hydroxynefazodone represents a circulating metabolite in human plasma after nefazodone administration [47,48]. Likewise nefazodone, the dealkylated metabolite m-CPP also undergoes further P450 2D6 mediated oxidation to *para*-hydroxy m-CPP [44,46,49].

Bioactivation products of nefazodone that are capable of covalent binding to hepatic proteins have recently been studied by Kalgutkar et al. [50]. Employing

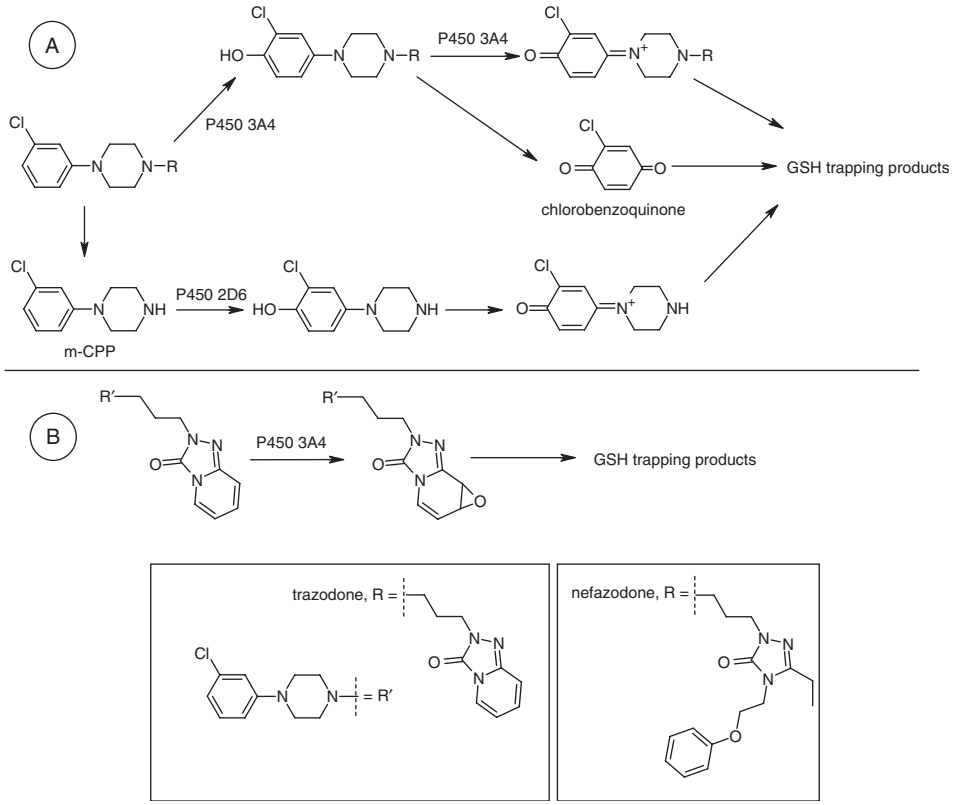


Figure 10 Antidepressant drugs nefazodone and trazodone and their bioactivation pathways in human liver. (A) Sequential metabolism *via para*-hydroxylation to the piperazine nitrogen and m-CPP formation with subsequent further two-step oxidation to glutathione-reactive imino-quinone species. (B) Trazodone-specific bioactivation involving aromatic epoxidation as suggested by Kalgutkar et al. [50,56].

hepatic biotransformation systems and mass spectrometric approaches, the authors identified stable products of reactive nefazodone metabolites after trapping with glutathione or glutathione-ethylester. Two-electron oxidation of *para*-hydroxy nefazodone gave rise to the corresponding quinine-iminium products that were efficiently trapped to yield the corresponding hydroquinone–sulfhydryl adducts. These were subsequently studied by liquid chromatography mass spectrometry for structural characterization. Structure of a glutathione adduct detected in nefazodone incubations with recombinant human P450 3A4 was consistent with the addition of glutathione to a monohydroxylated metabolite of nefazodone (Figure 10A). Kalgutkar et al. also report on the detection of a nefazodone-derived glutathione adduct that corresponds to the stable sulfhydryl conjugate of chlorobenzoquinone. Incubations of nefazodone in human liver microsomes fortified with the trapping reagent glutathione-ethylester or alternatively incubation of human liver microsomes with 2-chloro-1,4-benzoquinone afforded regioisomeric glutathione adducts consistent with the formation of the adducts depicted in Figure 10A. We applied a generic screening method for the detection of glutathione adducts in negative ion mode, based on the mass spectrometric detection of a specific glutathione-characteristic fragment ion, to human liver microsomal incubations of nefazodone fortified with glutathione. LC-MS/MS data indicate the presence of several unique sulfhydryl conjugates not formed in corresponding control incubations lacking the substrate. Besides the reported adducts derived from secondary oxidation of *para*-hydroxy nefazodone (affording adduct **P3**; Figure 11) and the sulfhydryl adduct derived form chlorobenzoquinone (adduct **P2**), an additional glutathione adduct unique to metabolically transformed nefazodone (adduct **P1**; Figure 11) with the characteristic isotope pattern indicative for the presence of a chloro atom was detected. The molecular weight of this adduct is consistent with a stable sulfhydryl adduct of the oxidized secondary nefazodone metabolite *para*-hydroxy m-CPP after secondary oxidation of *para*-hydroxy m-CPP.

The antidepressant trazodone has been associated with several case reports of idiosyncratic adverse drug reactions including hepatitis. Elevation of hepatic enzymes upon re-challenge with trazodone has led to the characterization of an idiosyncratic metabolic hepatic injury. Unlike for nefazodone, enzyme levels return to normal after discontinuation of trazodone therapy [51–53]. Similar to nefazodone, trazodone also undergoes P450 3A4-catalyzed aromatic hydroxylation para to the piperazine nitrogen leading to *para*-hydroxy trazodone [54,55]. Likewise nefazodone, subsequent secondary oxidation of *para*-hydroxy trazodone affords a reactive imino-quinone intermediate capable of covalently binding to protein sulfhydryl groups. Recently, trappable glutathione adducts of trazodone bioactivation products were described [56]. Given the close structural similarity to nefazodone, trazodone also produces m-CPP as a primary metabolite after dealkylation of the 3-chlorophenylpiperazine motif of the parent drug [57] (Figure 10A). Subsequent *para*-hydroxylation of m-CPP and secondary oxidation to the reactive imino-quinone species that ultimately can be trapped by cellular proteins or glutathione occurs similar to the bioactivation pathway described for nefazodone. In addition to the bioactivation pathways related to 1,4-Michael addition-type reactions *via para*-hydroxylation of the parent drug molecule or its

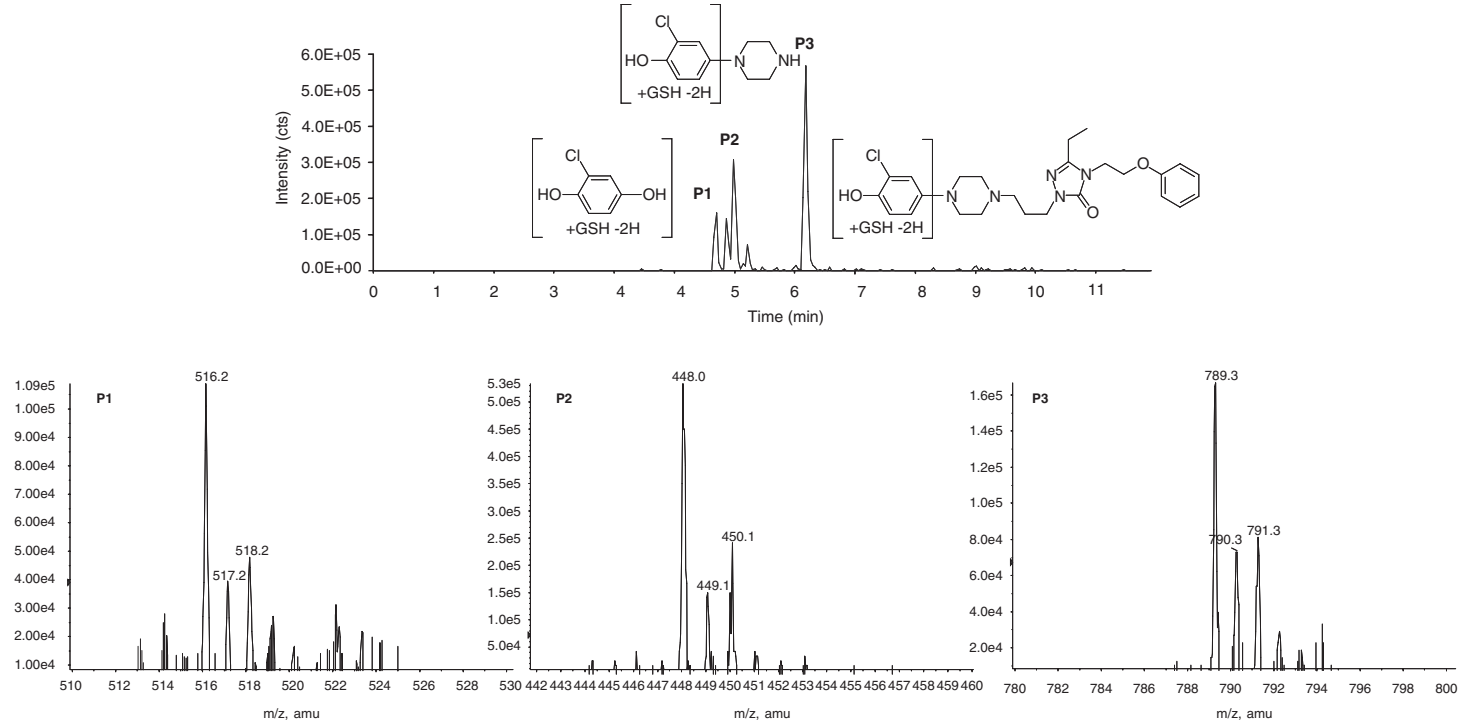


Figure 11 Detection of glutathione adducts of nefazodone in human liver microsomes. Extracted ion chromatograms of glutathione-conjugates detected by precursor ion screening of *m/z* 272 in negative ion mode (upper panel) and corresponding full scan enhanced resolution spectra of the deprotonated molecular ions of the indicated glutathione adducts P1, P2 and P3 (lower panel).

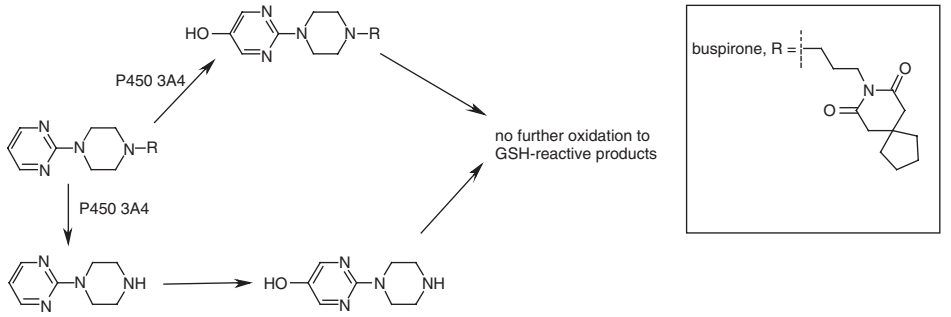


Figure 12 Metabolic transformation of the antidepressant buspirone into *para*-hydroxylated metabolites representing structural alerts for drug-bioactivation. Unlike its structural analogs nefazodone and trazodone, buspirone does not afford trappable, glutathione-reactive intermediates.

primary metabolite m-CPP, trazodone also undergoes epoxidation of the pyridinone motif affording a reactive intermediate that subsequently reacts with glutathione to afford a stable hydrated trazodone-thiol conjugate [56] (Figure 10B).

The antidepressant buspirone, which is not associated with hepatotoxicity like nefazodone or to a lesser extent trazodone, is similarly metabolized (Figure 12) *via* N-dealkylation [58–61]. Like m-CPP in case of nefazodone and trazodone, the primary buspirone pyrimidinyl-piperazine metabolite undergoes further oxidation of the corresponding *para*-hydroxy metabolite [59,60,62]. Kalgutkar et al. [50] have demonstrated that buspirone despite formation of *para*-hydroxylated metabolites of the parent drug and the major N-dealkylation product, does not afford stable SH-trapping products with glutathione. This finding suggests that a similar bioactivation as observed for nefazodone dose not occur in case of buspirone. The displacement of the 3-chlorophenyl ring in case of nefazodone with a pyrimidine ring in case of buspirone suggests that the proposed two-electron oxidation of *p*-hydroxybuspirone and its de-alkylated metabolite to the corresponding iminium-quinone ions is less favorable. However, the unequivocal proof that buspirone does not form significant reactive metabolites capable of covalent protein binding requires a thorough assessment employing radiolabeled drug.

Although the oxidative metabolic transformations of nefazodone, trazodone and buspirone are quite similar, reports on severe cases of idiosyncratic hepatotoxicity associated with drug treatment seem to be unique to nefazodone and to a lesser extent for trazodone but not associated with the clinical use of buspirone. Buspirone apparently does not form significant levels of glutathione-trappable reactive intermediates indicating that subsequent covalent binding to hepatic proteins does not represent a concern for this particular drug.

2.2. Drug-induced intrahepatic cholestasis

2.2.1. Cholestatic potential of troglitazone and other TZD drugs

Different hypotheses towards the mechanism(s) underlying the troglitazone-associated hepatotoxicity have been published. Several reports suggested a cholestatic mechanism to be involved in this liver injury [10] and a strong reduction of the

bile flow has been observed in isolated perfused rat liver [63]. Several cases of liver injury following concurrent troglitazone and glibenclamide treatment lead to the speculation that this latter drug, known to induce cholestasis in some patients [64] might enhance the probability for troglitazone-induced liver injury [12,65]. This cholestatic potential of troglitazone is further supported by *in vitro* and *in vivo* mechanistic studies conducted by our laboratories and others [66–68]. Troglitazone and to a greater extent its main metabolite, troglitazone-sulfate, inhibited the rat canalicular bile salt export pump (Bsep) (Figure 13) in a competitive manner with IC_{50} values of 3.9 and 0.4 μM, respectively [67]. The higher inhibition potential of troglitazone-sulfate in these rat liver membrane vesicles indicated the importance of this metabolite in contributing to the overall cholestatic potential of troglitazone. This was supported by studies in the isolated perfused rat liver (IPRL), where troglitazone triggered a strong decline in the bile flow in livers from male rats [63]. In this model, troglitazone was completely cleared from the liver perfusate, metabolized in the liver mainly to troglitazone-sulfate and -glucuronide and eliminated to a great extent into bile [67]. *In vivo*, in rats, troglitazone similarly interfered with the hepatic elimination of bile acids and induced a transient increase in plasma bile acid levels (Figure 14), while an accumulation of an intravenously administered radiolabeled taurocholate tracer was observed in liver tissue [67]. These results support the cholestatic mechanism of troglitazone and its metabolite troglitazone-sulfate at the canalicular pole of the hepatocytes, where Bsep is localized, as the site of interference with the hepatobiliary excretion of bile acids.

Several lines of evidence suggested that the hepatobiliary export of troglitazone and related metabolites might represent a rate-limiting step in the overall

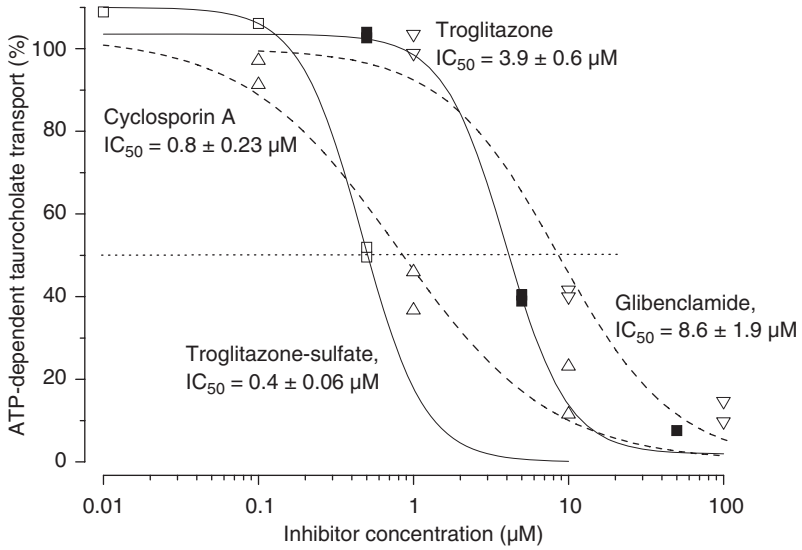


Figure 13 *In vitro* inhibition effects of troglitazone, troglitazone-sulfate and the two cholestatic model compounds cyclosporin A and glibenclamide on the ATP-dependent bile acid transport assessed using canalicular liver plasma membrane vesicles (cLPMV).

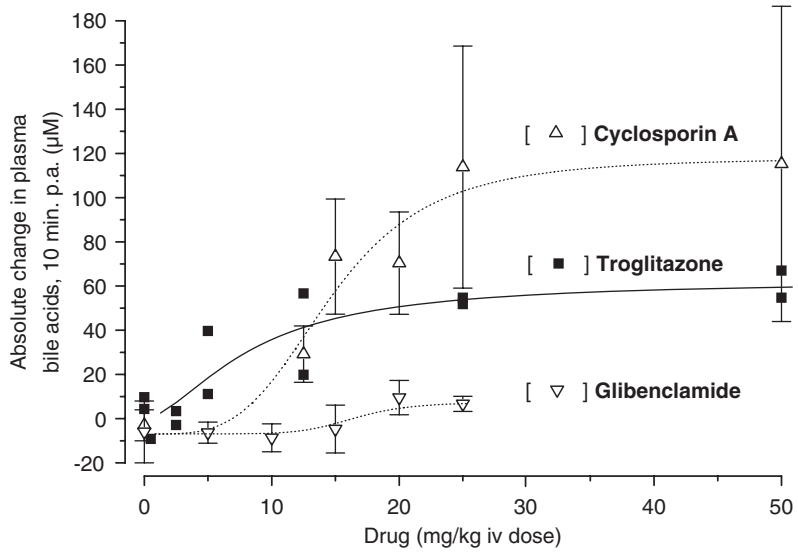


Figure 14 Acute *in vivo* effects of troglitazone and the two cholestatic model compounds on the plasma bile acid levels in rats. The changes in total bile acid levels (difference pre-dose and 10 min post-dose) are shown as mean of 3–4 rats per concentration.

elimination of troglitazone. In patients with hepatic impairment, troglitazone-sulfate was found to accumulate about four-fold in plasma, with a three-fold increased half-life [69]. In rats, troglitazone-sulfate accumulated as the major drug-related metabolite in liver tissue, reaching high intracellular concentrations [67]. The human BSEP appeared to be more sensitive for the compounds tested, especially for rosiglitazone, for which a 10-fold lower IC_{50} was observed using the human BSEP (0.4 µM; Table 1). Troglitazone inhibits the human bile salt transporter (BSEP) with an IC_{50} value of 2.3 µM, troglitazone-sulfate, inhibited BSEP with an IC_{50} of 4.2 µM. Also the low apparent inhibition of pioglitazone (IC_{50} of 0.4 µM) on the human BSEP is not in agreement with the clinical observations, as this compound is not associated with significant liver enzyme elevations. The dose, tissue distribution, metabolism and other factors have to be considered to explain this apparent discrepancy.

2.2.2. Cholestatic potential of nefazodone and analogs

The antidepressant Nefazodone (Serzone) was withdrawn from the market due to drug-mediated hepatic injury. On the basis of the clinical symptoms of the idiosyncratic hepatotoxicity including jaundice, increase in liver enzymes, high biliary elimination of nefazodone seen in animals, and the relative high therapeutic doses of nefazodone in the range of 200–400 mg/day, the potential for a cholestatic mechanism was studied in more detail [70]. *In vitro*, nefazodone caused a strong inhibition of the human bile salt transporter BSEP with an IC_{50} value of 9 µM, using membrane vesicles expressing BSEP. A similar inhibitory effect was observed in primary human hepatocytes. Nefazodone was furthermore found to interfere

Table 1 Comparison of clinical dose, human BSEP inhibition potential, bioactivation liabilities and clinical side effects for the medicines discussed in this article.

Compound	Clincal dose (mg per day)	BSEP inhibition potential IC_{50} (μM)[a]	Bioactivation proposed	Cytotoxicity in Human Hepatocytes	Clinical side effect (liver) described
Troglitazone	400–600	2.3	Yes	Yes	Severe, fatal liver injury, cholestatic
(TGZ-sulfate)		(4.2)			DDI potential with glibenclamide
Rosiglitazone	8	6.5	Yes	No	Rare
Pioglitazone	45	0.4	Yes	No	Rare
Nefazodone	200–400	9[b]	Yes	Yes	Severe, fatal liver injury, jaundice, bile duct proliferation, cholestasis
Trazodone	50–150	>100[b]	Yes	No	Isolated cases of idiosyncratic, reversible hepatitis
Buspirone	15–60	>100[b]	No	No	Rare

[a]IC_{50} value determined at one taurocholate concentration using Sf9 vesicles expressing BSEP.
[b]From reference [70].

with the bile acid elimination in sandwich cultured human hepatocytes. Cytotoxicity also was observed in human hepatocytes at nefazodone concentrations in the range of 10 μM. By inhibition of the metabolism, this could be associated with the concentrations of intact nefazodone rather than its metabolites. In rats, a transient increase in the serum bile acid level was observed at 1 h after oral drug administration. In this study, an approximately 60% increase in serum bile acids was evident that returned to normal levels 24 h after dosing. On the basis of these data, it is probable that the clinical hepatotoxicity of nefazodone is linked to the ability of the drug to interfere with the bile acid transport, primarily by an inhibition of the canalicular bile salt export protein (BSEP). Interestingly, for nefazodone also the formation of reactive metabolites and covalent binding to liver proteins, including P450 3A4, have been suggested as factors contributing to the hepatotoxicity of this compound [50].

Unlike nefazodone, the structural analogs trazodone and buspirone did not significantly inhibit the BSEP-mediated bile salt export at concentrations up to 100 μM. Also these safe drugs did not significantly affect protein synthesis as marker of cytotoxicty in primary human hepatocytes at concentrations up to 100 μM (Table 1).

2.3. Contributing risk factors

2.3.1. Drug-specific risk factors

Formation of quinone and quinone–methide-type reactive intermediates has been linked to the pro-oxidant activity of troglitazone in rat primary hepatocytes on cumene-hydroperoxide-induced lipid peroxidation and cytotoxicity. This effect was significantly higher for troglitazone as compared to other vitamin E analogs [71]. Cytotoxicity in hepatic cells and oxidative stress-inducing properties for TZD derivatives were dependent on the presence of the 6-hydroxychromane moiety, suggesting a link to this specific molecular structure of troglitazone. In addition, N1S1 rat hepatoma cells were more sensitive to troglitazone and troglitazone–quinone than to other TZD derivatives lacking the 6-hydroxychromane moiety [72]. Besides the potential direct cytotoxic effect of troglitazone-derived reactive metabolites on cellular structures, the drug was demonstrated to disrupt the mitochondrial function in rat hepatocytes [72,73]. Cellular toxicity studies pointed towards a direct toxicity of troglitazone or troglitazone-sulfate, while the metabolism by cytochrome P450 3A4 (which is also responsible for the formation of the reactive intermediate metabolites) rather represents a detoxification pathway [19,74]. In other studies, the conjugated metabolites troglitazone-sulfate and -glucuronide showed a lower potential for direct cellular toxicity [75,76] as compared to the parent drug molecule.

TZD-related bioactivation seems to represent a common property for these anti-diabetic drugs. For troglitazone, the predominant GSH adduct was not TZD-dependent. This troglitazone-specific bioactivation potential, its relative higher clinical dose as well as other risk factors such as hepatic oxidative stress, cholestasis potential and P450 inhibition and induction potentials [37] might contribute to the hepatotoxicity of this compound. All these risk factors might contribute to the multifactorial hepatotoxicity of this compound [18,19] in contrast to the lack of toxicity with the chemically related drugs rosiglitazone and pioglitazone.

Similarly, the antidepressant nefazodone was demonstrated to inhibit protein synthesis as an indicator of cytotoxicity in human hepatocytes at low substrate concentrations [70]. Trazodone that is associated with less frequent hepatotoxic events in the clinic, and also the safe drug buspirone did not produce significant cytotoxicity in human hepatocytes at substrate concentrations up to 100 μM. For this set of drugs, specific risk factors like drug-induced cytotoxicity and the potential to induce intrahepatic cholestasis in case of nefazodone seems unique for this compound and not associated with trazodone and buspirone. This difference and the lack of bioactivation processes in case of buspirone may contribute to the severe metabolic idiosyncratic reactions in case of nefazodone, which are less pronounced for trazodone and not associated with buspirone treatment.

2.3.2. Individual risk factors

Intrahepatic cholestasis has a genetic origin characterizing certain individuals more susceptible to adverse drug reactions than the average patient population. One form of progressive familial intrahepatic cholestasis (PFIC) is characterized by high serum and low biliary bile acid levels and normal serum γ-glutamyl transferase levels [77].

In one form a mutation in PFIC1, a P-type adenosine triphosphatase, is responsible for the cholestatic disease called "Byler disease" according to the Amish population descending from Jacob Byler where this form was discovered. Linked with a slightly different form of PFIC, PFIC2, mutations in BSEP were found to be responsible for this progressive liver disease [78]. An impaired activity of the hepatobiliary bile salt export pump leads to increased intracellular bile acid levels and intrahepatic cholestasis. Numerous mutations have been described in BSEP leading to different degrees of intrahepatic cholestasis. In PFIC2 patients, a functional expression of BSEP is typically missing, while in milder forms (benign recurrent intrahepatic cholestasis, BRIC) different mutations in BSEP lead to proteins with lower functional activity. Such a mutation was described in a patient with BRIC and the BSEP protein bearing the respective mutation showed *in vitro* a significantly reduced taurocholate transport activity [79]. Such BSEP transport proteins with reduced activity, as found in benign recurrent intrahepatic cholestasis, might render a patient more sensitive and in combination with cholestatic drugs lead to a significant intrahepatic cholestasis. Although the molecular properties of troglitazone or nefazodone do not represent a cause for hepatotoxic events per se, it seems probable that certain individuals are more susceptible to troglitazone-induced hepatotoxicity, especially in disease populations. For example, diabetic patients with a history of cholestasis are especially at risk from troglitazone [18,80].

Typical daily troglitazone doses in man are in the range of 400–600 mg, producing troglitazone plasma concentrations of 1–2 μg/ml (Table 1; [81,82]). A quantitative extrapolation of the cholestatic effect observed in rats to man is not possible, due to species-specific differences in the process of bile formation and in the overall disposition of troglitazone. However, the processes involved in the hepatobiliary metabolism and export of the absorbed fraction of troglitazone, represent potential targets for drug-interactions and regulative changes. Therefore, troglitazone might induce intrahepatic cholestasis in combination with other cholestatic drugs, other diseases, or pharmacogenetic liabilities, thereby contributing to the hepatotoxicity observed in some patients treated with troglitazone. Simvastatin [11] and lisinopril [10], both drugs known to induce cholestatic side effects, were co-medicated with troglitazone in patients developing signs of liver toxicity. Another report described three out of four cases of troglitazone-induced fulminant hepatitis where glibenclamide has been co-medicated with troglitazone. An interaction of the two drugs was suspected without further elaborating on a possible mechanism [12]. Co-medication of potentially cholestatic drugs with troglitazone might increase the incidence in patients to develop liver toxicity.

3. Proactive Approaches to Drug Safety

3.1. Reducing drug bioactivation *via* reactive metabolites assessment

Although the causal link between reactive metabolite formation and clinically manifested ADRs is mainly anecdotal in nature, an early assessment and possible

avoidance of bioactivation reactions has become an integral part of the drug discovery and development process. Strategies for the preclinical characterization of bioactivation liabilities of new chemical entities (NCEs) have been developed in recent decades and are being pursued in many pharmaceutical companies [83,84]. Reactive metabolite formation and covalent binding alone rarely seem to be the cause of idiosyncratic drug reactions. However, it is believed that the risk for adverse events due to covalent binding can be significantly reduced by avoiding chemical functionalities known to be susceptible to reactive metabolite formation. This approach has become the gold standard in pharmaceutical industry. Preclinical tools for the assessment of metabolism with regard to reactive intermediate formation are applied to assess liabilities associated with covalent binding. These assays serve as confirmatory assays and employ the detection of radiolabeled drug or metabolites covalently bound to hepatic proteins [85]. Nowadays this more formalized process might also help establish structure activity relationships for such liabilities at least for local systems and allows medicinal chemists to find compounds optimized with regard to minimized reactive metabolite formation that can be supported by early metabolite identification efforts. Although few cases of successful predictions of reactive metabolite formation using structure activity relationship (SAR) and adverse drug reactions exist [86,87], the most commonly used approach would include the recognition of structural alerts associated with reactive metabolites formation by educated guess. This approach builds on the expertise of medicinal chemists and drug metabolism specialists to prioritize compounds for testing in the appropriate *in vitro* tools for the characterization of metabolic liabilities. Higher throughput *in vitro* methods such as the cytochrome P450 time-dependent inactivation assay and the screening for drug–glutathione adducts have emerged over the last decades and made it possible to address bioactivation liabilities experimentally early in drug discovery [85,88–91]. The mass spectrometric detection of glutathione adducts is commonly conducted by applying the constant neutral loss screen for the loss of pyroglutamate in the positive ion mode [89]. Likewise, the detection of glutathione adducts in the negative ion mode based on the characteristic fragment ion of glutathione at m/z 272 [91], that is common for all glutathione adducts independent of the attached drug-related motif, can be employed for a generic compound-independent screening for drug–glutathione adducts. The latter assay is routinely conducted as a screening method in our laboratories and proved more specific and producing less background as compared to the screening method based on the detection of a common neutral fragment in positive ion mode. Although targeted detection of drug–glutathione adducts based on proposed bioactivation processes has been successfully and with high specificity applied to a variety of problematic drugs [35], the generic screening method allows for the unbiased detection of glutathione trapping products without prior knowledge of actual metabolic transformation steps. Still, the most promising approach for the development of safe and efficacious new drugs is the discovery of highly potent and selective molecules because many adverse drug reactions involving reactive metabolite formation have been attributed preferentially to those medicines that are used at higher doses [8,92,93].

3.2. Reducing the potential of drug candidates to induce intrahepatic cholestasis

The potential of certain drugs to induce intrahepatic cholestasis has been outlined above and the mechanism(s) by which these compounds interfere with the hepatobiliary elimination of bile acids, mainly by inhibition of BSEP by the drug or major drug metabolites, is well documented for many of these cholestatic compounds. Drugs which are used at relatively high doses and for which biliary excretion of parent drug or metabolites represents a major elimination pathway might be particularly prone to interfere with bile acid elimination.

The *in vitro* inhibition of the cannalicular Bsep/BSEP can be easily determined using appropriate *in vitro* systems, such as assessing the ATP-dependent taurocholate transport in liver plasma membrane vesicles or membrane vesicles expressing the rat/human transporter [67,70,75,94]. Special attention has to be given to include the relevant major drug metabolites in these studies. A high-speed screening system has been reported recently, ATP-dependent uptake was measured using BSEP expressing membrane vesicles and 96-well filtration plates [95]. The same authors also assessed the vectorial transport of taurocholate using double transfected cells [95]. The polarized LLC-PK1 cells expressed the human uptake transporter Na-taurocholate cotransporting polypeptide (NTCP) and the export transporter BSEP at the respective membrane and were grown as monolayer on membrane inserts and fluorescent bile acids were used as substrates. The inhibition of the bidirectional transport by a group of cholestatic model compounds was assessed. The drawback of this system is the evaluation of the quantitative inhibition potential of the exporter BSEP, as the intracellular concentration of the test compound is unknown. For the evaluation of the cholestatic potential of metabolized compounds, the relevant metabolites have to be assessed separately, as these cells are metabolically not competent.

In addition to the *in vitro* inhibition studies with Bsep/BSEP, it is important to consider the *in vivo* effect, including tissue (liver) distribution kinetics and metabolism of the test drug. Animal models are used for this purpose with the obvious limitations of species differences in transport and metabolism. An acute cholestasis model in rats was used to characterize cholestatic compounds assessing the effects of intravenously administered compounds on the plasma bile acid level [66,67]. A transient increase in bile acids can be observed for cholestatic compounds, allowing a ranking of the *in vivo* cholestatic potential. Results of the *in vitro* Bsep inhibition are shown in correlation to the *in vivo* cholestatic potential in rats for a series of molecules within one chemical class along with the corresponding results of the cholestatic reference compounds cyclosporin A and glibenclamide (Figure 15). This approach allows for the selection of compounds with a low cholestatic potential and can be systematically applied early on in the drug research and development process.

4. Summary and Conclusions

The examples of troglitazone and nefazodone (and to a lesser extent trazodone) highlight the interplay between drug metabolism properties of a drug

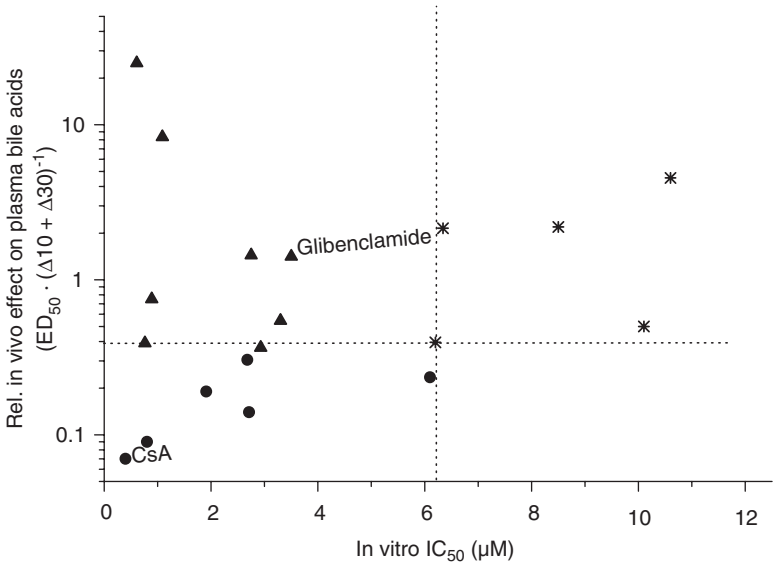


Figure 15 *In vitro* and *in vivo* cholestatic potential of reference compounds (cyclosporin A and glibenclamide) and representatives of one chemical series. The *in vitro* cholestatic potential was determined as the IC_{50} of Bsep inhibition in cLPMV, while the *in vivo* effect represents the increment of plasma bile acids increase in rats. Compounds with both, low *in vitro* (high IC_{50} value) and *in vivo* cholestatic potential (stars) were selected for further characterization.

with the complex physiological processes that lead in most cases to multi-factorial drug-induced hepatotoxicity. Although toxic properties are associated with certain chemical motifs, the mechanisms by which such problematic drugs elicit their toxicities carry strong host-associated components that are not predictable. Thus, mechanistic toxicological investigations and the in-depth biochemical understanding of preclinical safety findings in multiple species will help in the future to provide more predictive insight to enable the development of effective and safe drugs for humans. Reactive metabolite formation and covalent binding to proteins might represent one mechanism contributing to the hepatic toxicity observed in patients with troglitazone, nefazodone and to a lesser extent with trazodone but apparently not with rosiglitatzone and pioglitazone. A combination of multiple mechanisms, namely cholestasis leading to increased drug levels in hepatocytes, mitochondrial toxicity induced by troglitazone, nefazodone and/or bile acids, reactive metabolite formation and covalent protein binding at high doses associated with antigen presentation and stimulation of an immune-response might be triggered by troglitazone, nefazodone and trazodone or their respective metabolites. Drug-induced intrahepatic cholestasis might thereby increase the individual susceptibility to develop idiosyncratic metabolic hepatotoxicity. This could fit with the proposal of haptenization associated low-grade toxicity (danger signal) needed for a significant immune reaction [21,22,96].

Recent studies have provided evidence that sulfhydryl-reactive intermediates of nefazodone and trazodone are capable of covalent binding to tissue proteins. This

bioactivation liability seems unique to nefazodone and trazodone and not associated with the safe analog buspirone. In case of the thioazolidinedione drugs troglitazone, rosiglitazone and pioglitazone studies from our group and others have revealed that rosiglitazone and pioglitazone are also prone to form reactive intermediates at higher substrate concentrations, suggesting that covalent binding to liver proteins may also be expected for these two compounds. In human liver microsomes at low drug concentrations the formation of GSH adducts was evident for troglitazone *via* TZD-independent biotransformation but almost not detectable for rosiglitazone and pioglitazone. Although the relative contribution of less abundant reactive intermediates to hepatotoxic events remains unknown, these findings suggest that the observed hepatotoxic potential of troglitazone might be associated with the formation of reactive intermediates not related to the TZD-moiety. Bioactivation of all three TZD drugs has been demonstrated, however the high dose associated with troglitazone therapy in contrast to low doses warranted for rosiglitazone and pioglitazone efficacy (Table 1), puts troglitazone at high risk for metabolic idiosyncratic hepatotoxicity in conjunction with its additional risk factors.

Preclinical methods exist to early address bioactivation liabilities of new chemical entities as well to mechanistically investigate their potential to inhibit bile acid transport proteins *in vitro* or to address the *in vivo* cholestatic potential in rats for a series of molecules within one chemical class. However, the interpretation of such data must be performed with care not to exclude promising new medicines from clinical development. It is tempting to assume this hypothesis as probable underlying mechanistic hypothesis for the failure of these two model drugs. Still, the accumulated evidence does not provide a formal proof. The molecular properties of the non-hepatotoxic drugs rosiglitazone and pioglitazone with respect to human bile acid transporter (BSEP) inhibition are similar to the problematic drug troglitazone; also similar bioactivation pathways of the TZD have been characterized. Many other mechanisms such as oxidative stress have been implicated in drug-induced liver injury and the effects of covalent binding would only become effective in conjunction with these other risk factors. Finally the clinical dose, tissue distribution, metabolism and other factors such as interfering co-medications and polymorphic enzyme/transporter expression have to be considered to assess the potential clinical risk for idiosyncratic metabolic liver injury.

It is our current belief that the proposed preclinical tools are of great value to address proactively the potential for adverse drug reactions by identifying problematic molecular properties of development compounds. Applied early during the lead identification and compounds optimization stage of drug discovery, this approach will help selecting "low risk" drug candidates devoid of such potentially adverse properties. For highly potent, selective drugs that exert clinical efficacy at low doses (sub 10-50 mg) apparently the risk for metabolic idiosyncratic hepatotoxicity is low [8,23,92]. This assumption even seems to hold true despite bioactivation potential or other potentially adverse molecular properties as evidenced for the examples rosiglitazone, pioglitazone and trazodone as opposed to the "problematic" drugs troglitazone and nefazodone (Table 1). However, none of such molecular properties would be quantitatively predictive for the development of late-stage clinical hepatotoxicity. Given this caveat, the true value of

the early characterization of potentially adverse drug properties is to serve as an "eye-opener" that should guide the decisions on compound progression without eliminating promising new drug candidates based on individual, potentially adverse molecular properties. In conjunction with the preclinical and clinical safety evaluation of new drug molecules, these findings may help to allow for an informed risk assessment and will guide later stages of development and safe market introduction [97].

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CHAPTER 3

Glucuronidation-Dependent Toxicity and Bioactivation

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Contents

1. Introduction

Glucuronidation, catalysed by the UDP-glucuronosyltransferase (UGT) enzymes, is a significant metabolic pathway for most endogenous and exogenous

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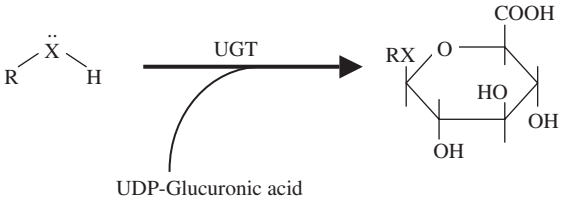

Figure 1 Schematic representation of the reaction catalysed by UGT enzymes. Functional groups commonly conjugated with glucuronic acid include −OH, −COOH, $-NH_2$, −NH, $-NR_2$ and −SH groups.

compounds. The UGTs catalyse the conjugation of compounds possessing a nucleophilic acceptor group with glucuronic acid (Figure 1), a relatively bulky, hydrophilic moiety, whose carboxylic acid functional group is ionised at physiological pH. Thus, forming metabolites with significantly different chemical and biochemical properties to the parent aglycone and, in most cases, with significantly decreased affinity for the receptors or enzymes responsible for the biological activity of the parent compound. In addition, due to the presence of the anionic glucuronic acid moiety, glucuronide conjugates are substrates for the human MRP2 (rodent Mrp2) efflux transporter localised on hepatic canalicular membranes and renal tubular brush border membranes, facilitating their transport into bile and urine, respectively. Due to their hydrophilicity, glucuronide conjugates are unlikely to be passively reabsorbed into the epithelial cells lining the biliary or urinary tracts, thus glucuronidation coupled with transporter-mediated efflux (and renal glomerular filtration) facilitates the excretion of endobiotics and xenobiotics from the body. Consequently, UGTs have for the most part been considered as enzymes facilitating the deactivation, detoxification and excretion of both endogenous and xenobiotic compounds. However, it has become increasingly evident that this is not always the case. Like the metabolites formed by the cytochromes P450 (CYP), glucuronide conjugates may retain, have enhanced or different pharmacological activity compared to the parent aglycone (see Section 2). Importantly, UGTs may catalyse bioactivation reactions, that is the metabolism of chemically stable compounds to electrophilic reactive metabolites able to damage intracellular macromolecules. Additionally, unlike the metabolites formed by CYPs, the affinity of glucuronide conjugates for efflux transporters, may also allow them to act as "shuttles" for proximate or ultimate carcinogens and/or toxins to the biliary-intestinal tract, kidneys and bladder.

This review complements and updates previous reviews on glucuronidation-dependent bioactivation [1,2], focusing on examples of glucuronidation as a toxification and bioactivation pathway, and as a metabolic pathway that facilitates translocation of toxins to the gastrointestinal and urinary tracts. Several excellent recent reviews are available describing the distribution and substrate specificities of the UGT enzyme families, as well as the important role of glucuronidation as a detoxification pathway [3–5].

2. Pharmacologically Active Glucuronide Conjugates

A number of glucuronide conjugates are now known to possess significant pharmacological activity, either therapeutic or toxicological. For example morphine-6-glucuronide is a potent μ-opioid receptor agonist and analgesic [6,7]; α-hydroxymidazolam glucuronide has sedative activity and binds to the benzodiazepine receptor [8]; digitoxin glucuronide and digoxin glucuronide have positive inotropic effects [9], the glucuronide conjugates of retinoic acid and retinol have enhanced retinoid activity [10] and mycophenolate acyl glucuronide promotes leukocyte cytokine release [11]. The proinflammatory activity of mycophenolate acyl glucuronide has been proposed to contribute to the gastrointestinal side effects of the immunosuppressant mycophenolic acid [11], in addition accumulation of morphine-6-glucuronide and α-hydroxymidazolam glucuronide during renal dysfunction may contribute to the respiratory side effects of morphine (reviewed in [12]) and excess sedation by midazolam [8].

Several glucuronide conjugates are cholestatic, including steroid D-ring glucuronides [13], the bile acid conjugate lithocholate-3-*O*-glucuronide [14,15] and the xenobiotic conjugate harmol glucuronide [16]. The most potent of the cholestatic steroid D-ring glucuronides is estradiol-17β-D-glucuronide, which significantly decreases *in vivo* bile flow in rats at i.v. doses as low as 8.5 μmol/kg [17] and significantly retards the elimination of indocyanine green from plasma of rhesus monkeys at an i.v. dose of 5.5 μmol/kg [18]. Steroid D-ring glucuronides are thought to contribute to cholestasis and jaundice in women during pregnancy and during chronic high dose use of oral contraceptives (reviewed in [13,17]). Estradiol-17β-D-glucuronide is an Mrp2 substrate [19], and its cholestatic effect in rats is dependent on Mrp2 expression and function [20]. Similarly, the cholestatic effect of lithocholate-3-*O*-glucuronide in rats is also dependent on Mrp2 expression and function [21]. However, the development of cholestasis does not appear to be related to the biliary concentrations of either glucuronide [20,21], suggesting that accumulation of the conjugates in bile is not necessary for cholestasis, but rather that the interaction of the glucuronide conjugates with Mrp2 is important for the development of cholestasis. Estradiol-17β-D-glucuronide was recently shown to induce endocytic retrieval, or internalisation, of Mrp2, as well as the canalicular bile acid transporter, Bsep and other canalicular membrane proteins in rats [22]. The effects of lithocholate-3-*O*-glucuronide on transporter localisation have not been described, however its cholestatic effects in rats are associated with morphological changes in the canalicular membrane [23].

Some glucuronide conjugates have been reported to possess choleretic properties, including endogenous conjugates such as steroid A-ring glucuronides [13,17], as well as xenobiotic glucuronide conjugates such as valproic acid glucuronide [24] and 4-hydroxyacetophenone-4-*O*-β-glucuronide [25]. The choleretic effects of valproic acid glucuronide and hydroxyacetophenone glucuronide in rats also depend on expression and function of Mrp2 protein and have been proposed to be due to the osmotic activity of the conjugates in bile [24,25].

3. Chemically Reactive Glucuronide Conjugates

Although most glucuronide conjugates are chemically stable, the conjugation of glucuronic acid with carboxylic acid or hydroxamic acid functional groups (Figure 2) results in the formation of electrophilic, chemically reactive metabolites that have been implicated in a number of toxicological processes including enteropathy, hypersensitivity and carcinogenesis.

Acyl (i.e. carboxyl- or ester-linked) glucuronides are formed by a large number of substrates including endogenous compounds such as bilirubin, retinoic acid, leukotrienes, bile acids and fatty acids, as well as xenobiotics encompassing a wide variety of medicinal classes, such as non-steroidal anti-inflammatory drugs (NSAIDs), hypolipidaemic agents ("fibrates"), hypoglycaemic agents ("glitazars"), immunosuppressants (mycophenolic acid), diuretic agents (furosemide) and anticonvulsants (valproate).

The *N*-*O*-linked glucuronides of hydroxamic acids are mainly secondary metabolites formed following phase I oxidation and/or phase II acetylation of industrial arylamines such as 2-acetylaminofluorine, 4-aminobiphenyl and benzidine. Many arylamines are products of cigarette smoke, combustion and the chemical, dye and rubber industries and are human and animal carcinogens. Some hydroxamic acids are also metabolites of compounds that have been used clinically, such as the analgesic, phenacetin.

3.1. Acyl glucuronides

The chemical reactivity of acyl glucuronides largely reflects the electrophilicity of their ester functional group, which is susceptible to non-enzymatic nucleophilic transacylation reactions (Figure 3). Thus, acyl glucuronides are readily hydrolysed under mild alkaline conditions [26–29] following transacylation by free hydroxyl anions. The ester group can also undergo transacylation with nucleophiles such as glutathione [30–33], or nucleophilic centres within proteins [34,35], to form covalently bound aglycone-protein adducts and liberate glucuronic acid. In addition, intramolecular transacylation reactions with the hydroxyl groups of the glucuronic acid moiety result in cleavage of the 1-*O*-β-glycosidic bond and intramolecular migration of the aglycone moiety to the C-2, -3 or -4 positions on the sugar [36–39] (Figure 3). The resulting rearranged conjugates are not substrates for β-glucuronidase [26,29,40] and are thus resistant to enzymatic breakdown

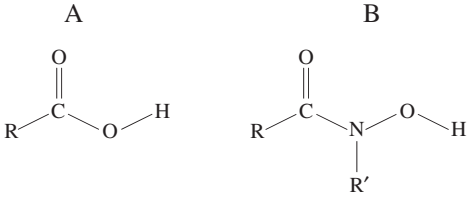

Figure 2 Chemical structures of (A) carboxylic acids and (B) hydroxamic acids.

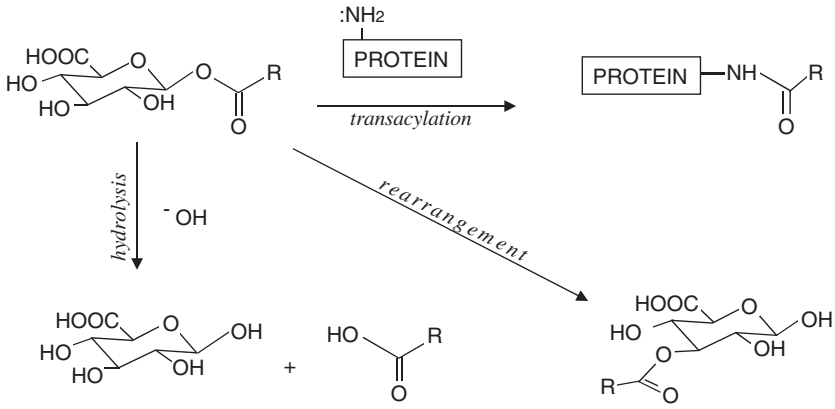

Figure 3 Non-enzymatic reactions of the biosynthetic 1-*O*-*β* acyl glucuronide conjugates arising due to the electrophilicity of the ester functional group. For illustrative purposes, only the C-3 rearrangement isomer is shown.

compared to the UGT-catalysed 1-O-*β*-conjugates. Importantly, intramolecular rearrangement re-exposes the hemiacetal function of glucuronic acid, unmasking the inherent reactivity of the sugar moiety and allowing a series on non-enzymatic reactions typical of monosaccharides, including anomerisation [36,37,41], glycation of endogenous macromolecules [34,35] and further glycoxidation to form advanced glycation endproducts (AGEs) [42], as well as autoxidation generating free radicals [43] (Figure 4). Although the chemical reactivity of acyl glucuronides was first described for the glucuronide conjugates of bilirubin [44,45], most recent studies have focused on the acyl glucuronides formed by carboxylic acid drugs, and several excellent reviews have been published in this area [46–50].

In aqueous solution at physiological pH and temperature, the biosynthetic 1-*O*-*β*-conjugates readily degrade as a result of intramolecular rearrangement, with half-lives ranging from 0.4 h (for the glucuronides of the NSAIDs tolmetin [51] and zomepirac [27]) up to 40–72 h (for the glucuronides of the hypolipidaemic agent gemfibrozil [52] and the antiepileptic agent valproic acid [53]). In aqueous solutions at physiological pH and temperature, hydrolysis of the biosynthetic conjugate is only a minor pathway of *in vitro* degradation, with significant re-formation of aglycone only detectable after the 1-*O*-*β*-conjugate has practically entirely rearranged [27,38,39,51]. However, the rate of *in vitro* degradation increases with increasing pH, and whilst at mild alkaline pH (8–9) intramolecular rearrangement remains the predominant reaction [26,28,29,39,52], at higher pH values there is complete hydrolysis of both the 1-*O*-*β*- and rearranged conjugates [26,28,29].

Since the relative *in vitro* degradation of the 1-*O*-*β*-conjugates reflect their potential to undergo intramolecular nucleophilic transacylation, it may serve as a measure of their relative reactivity and reflect their potential to covalently modify endogenous macromolecules and cause cellular damage. Several groups have investigated structure activity relationships that may be predictive

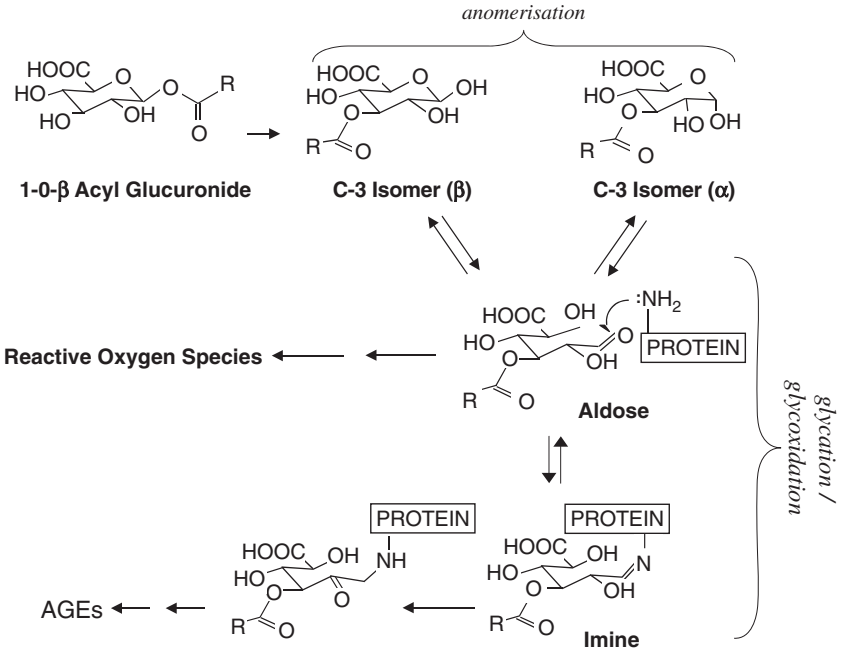

Figure 4 Non-enzymatic monosaccharide-like reactions of acyl glucuronide conjugates arising following intramolecular rearrangement exposing the hemiacetal function of the glucuronic acid moiety. For illustrative purposes, only the C-3 rearrangement isomer is shown.

of acyl glucuronide reactivity. Such studies have shown that steric effects significantly influence reactivity, with bulky functional groups near the carboxyl carbon hindering intramolecular rearrangement [37,54]. In addition, reactivity may also be determined by electronic properties such as the dipole moment of the molecule and the nucleophilicity of the carbonyl carbon [37]. The extent of formation of rearrangement isomers *in vivo* is unclear. Due to the presence of esterases and β-glucuronidase, hydrolysis becomes a significant breakdown pathway for the 1-*O*-β-conjugates *in vivo* [55,56], in situ [57,58] and *in vitro* in biological solutions [41,52,59–61]. However, in studies which have immediately stabilised biological samples to prevent *ex vivo* degradation of the conjugates, acyl glucuronide rearrangement isomers of several NSAIDs and valproic acid have been detected in plasma, bile and/or urine of humans, rhesus monkeys and rats administered the parent aglycone [62–69]. The rearrangement isomers accounted for up to 6% of the total plasma acyl glucuronide of ibufenac in rhesus monkeys [69], up to 33% of the total acyl glucuronide of zomepirac excreted in rat bile and urine [62], approximately 4% of the total ibuprofen glucuronides excreted in urine in elderly volunteers [68] and approximately 4 and 22% of the total acyl glucuronide of valproic acid excreted in urine of patients with normal or diminished hepatic and renal function, respectively [67].

3.2. Hydroxamic acid *N-O*-linked glucuronides

The reactivity of *N-O*-glucuronide conjugates of hydroxamic acids was first described in the 1960s for a number of carcinogenic industrial arylamines and several reviews describing their reactivity were published in the 1970s and 1980s [70,71]. Based on work with 2-acetylaminofluorene, at neutral pH the *N-O*-glucuronide conjugates of arylhydroxamic acids are thought to undergo heterolytic decomposition to form the electrophilic *N*-acetyl-*N*-arylnitrenium ion [71] (Figure 5). However, like carboxyl-linked conjugates, aryl hydroxamic acid *N-O*-glucuronides are also unstable under mildly alkaline conditions and, may undergo deacetylation, generating highly reactive electrophilic arylhydroxylamine *N-O*-glucuronides [70–72] (Figure 5). Indeed, chemically synthesised *N*-OH-2-aminofluorene *O*-glucuronide was shown to be much more reactive than the biologically formed *N*-OH-2-acetylaminofluorene *O*-glucuronide [73]. Interestingly, UGTs do not appear to catalyse the formation of *N-O*-linked glucuronide conjugates of arylhydroxylamines, and these species may be formed only as decomposition products of arylhydroxamic acid *N-O*-linked glucuronides. At physiological pH and temperature, the half-lives of the *N-O*-glucuronides of

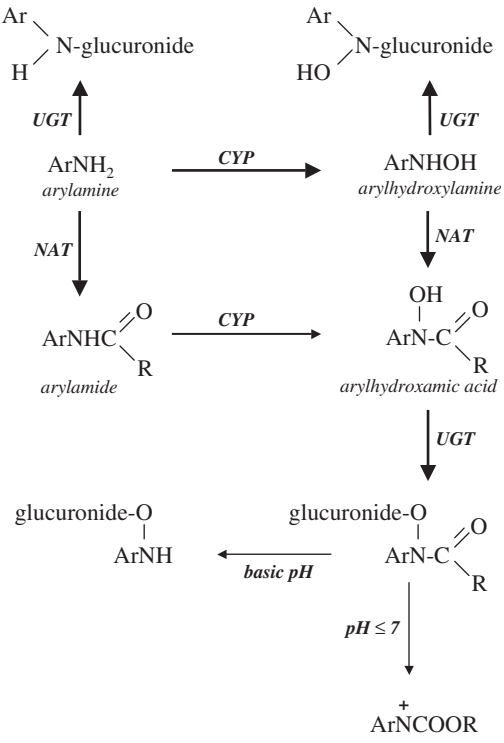

Figure 5 Structures and enzyme- or pH-catalysed metabolism of arylamines, arylhydroxylamines, arylamides and arylhydroxamic acids (Ar=aryl, R=methyl, CYP=cytochrome P450 enzyme, NAT=*N*-acetyltransferase enzyme, UGT=UDP-glucuronosyltransferase enzyme).

arylhydroxamic acids in aqueous buffered solutions range from >24 h for *N*-OH-*N,N′*-diacetylbenzidine *O*-glucuronide [74] to 2 h and 68 min for *N*-OH-*N*-acetylbenzidine *O*-glucuronide [74] and *N*-OH-*N*-acetyl-4-aminobiphenyl *O*-glucuronide [75], respectively, suggesting that the aryl chemical structure significantly affects reactivity.

The formation of *N*-linked glucuronide conjugates of arylhydroxylamines and arylamines is also catalysed by UGTs and, although acid-labile, they are not considered bioactivation products [74–76], but are thought to influence the carcinogenicity of arylamines and heterocyclic amines by acting as shuttles for CYP and *N*-acetyltransferase bioactivation products to the bladder and gastrointestinal tract (see Section 6.2).

4. Glucuronidation-Dependent Protein Damage

4.1. Acyl glucuronides

The ability of acyl glucuronides to form covalently bound adducts with endogenous macromolecules is now well documented and has been the subject of several excellent reviews [47,49,50]. The direct involvement of acyl glucuronides in the covalent modification of endogenous macromolecules has been demonstrated: (i) *in vitro*, by direct exposure of plasma proteins [59,77–81] or tissue microsomal protein [61,82] to acyl glucuronides, or by incubation of microsomal protein with carboxylic acid drugs in the presence and absence of glucuronidation co-factors [83,84]; (ii) *in situ*, using cultured hepatocytes exposed to carboxylic acid drugs in the presence and absence of glucuronidation inhibitors [85–87], or using control and UGT-expressing cell lines incubated with carboxylic acid drug [88,89] and (iii) *in vivo*, in rats administered carboxylic acid drugs with or without pre-treatment with glucuronidation inhibitors [90,91]. Elucidation of the chemical mechanisms of adduct formation has come primarily from *in vitro* studies utilising: (i) radiolabelled acyl glucuronide, labelled either on the drug or glucuronic acid moiety [59,92]; (ii) imine trapping reagents to investigate covalent binding *via* the glycation pathway [83,93–95] or (iii) more recently, with mass spectrometric analysis of covalently modified proteins and peptides [30–35,96–99].

In the simplest of the mass spectrometric studies, the 1-*O*-β acyl glucuronides of clofibric acid, diclofenac, naproxen and 2-phenylpropionic acid were incubated directly with the tripeptide glutathione in buffered solutions at physiological pH and temperature [30–33]. The conjugates were shown to undergo non-enzymatic transacylation reactions with the nucleophilic –SH moiety of glutathione, forming the corresponding glutathione conjugate and liberating glucuronic acid. In these studies, there was no evidence of glycation products in which glutathione was covalently bound *via* the reactive open-ring glucuronic acid moiety of the rearrangement isomers. In addition, no reaction was reported with the $-NH_2$ group of the glutamate residue of glutathione. The observed direct nucleophilic substitution of the 1-*O*-β acyl glucuronides by the glutathione thiol group is consistent with its greater nucleophilicity compared to amine groups.

In similar experiments with the dipeptide Lys-Phe, the acyl glucuronides of seven carboxylic acid drugs were shown undergo glycation reactions with the $-NH_2$ group of the lysine residue, with relative rates of glycation proportional to the relative rates of intramolecular rearrangement of the glucuronides [96]. Additionally, the degree of substitution of the carbon α to the carboxyl function appeared to determine reactivity in part, consistent with the effects of steric hindrance on intramolecular rearrangement of acyl glucuronides. Comparing the different mechanisms of covalent binding between glutathione and the Lys-Phe dipeptide, it appears that, in the presence of relatively strong nucleophiles (e.g. sulphydryl groups) direct nucleophilic substitution of the 1-*O*-β-conjugate takes place, whilst with weaker nucleophiles (e.g. amine groups) glycation *via* the rearrangement isomers may become more significant.

Consistent with a role for both binding mechanisms, *in vitro* incubation of the 1-*O*-β acyl glucuronides of the NSAIDs tolmetin and benoxaprofen with albumin at physiological pH and temperature has been shown to result in the formation of covalently bound adducts *via* both glycation of lysine residues, and direct nucleophilic transacylation by the amine group of lysine, the hydroxyl group of serine and the guanidine groups of arginine [34,35]. In these studies, the relative importance of glycation versus direct nucleophilic substitution reactions appeared to be related to the ratio of acyl glucuronide to albumin, with glycation predominating at low (physiological) ratios. However, interpretation of these results is difficult, since albumin reversibly binds carboxylic acid drugs and their glucuronide conjugates. Hence, the reversible ligand-protein binding affinities and the nucleophilic amino acid residues present at the binding sites may also affect the mechanism and extent of adduct formation [52,92,100,101].

In addition to albumin, acyl glucuronides have also been shown to form covalently bound adducts with a number of intracellular proteins *in vitro* [61, 82–89,102,103]. These include a number of hepatic microsomal proteins, localised primarily in the canalicular membrane [84] (of which only dipeptidyl peptidase IV has so far been identified as a possible target [104,105]), UGT proteins [89], tubulin [102] and superoxide dismutase [103]. The chemical mechanisms of adduct formation with intracellular proteins are unclear, although, there is evidence for the glycation mechanism in the formation of covalently bound adducts with hepatocyte proteins [57,83]. In each case, *in vitro* protein adduct formation has been associated with decreased protein function [89,102,103,105].

Consistent with their ability to undergo monosaccharide-like glycation reactions, *via* formation of an imine (or Schiff base) (Figure 4), acyl glucuronide-derived protein adducts have also been shown to undergo further *in vitro* reactions to form AGEs, similar to monosaccharides [42]. Interestingly, *in vitro* acyl glucuronide-mediated AGE formation appears to be more rapid than that of monosaccharides [42].

A large number of carboxylic acid compounds have also been shown to form covalently bound protein adducts *in vivo*, in humans and rodents. Adducts have been detected with plasma [52,53,68,81,82,106–116], liver [82,84,91,110,115,117–120], kidney [82,115,117] and intestinal proteins [117,121]. For the most part, identification of the protein adducts in these studies has relied on *in vivo*

administration of parent drug and collection of target tissues/proteins followed either by protein precipitation with extensive washing to remove non-covalently bound drug, hydrolysis of the adduct and chromatographic quantitation of the liberated parent drug; or by SDS-PAGE protein separation and immunoblotting using anti-aglycone antibodies. Unfortunately, neither of these methods provides structural information on how the adducts are formed, since they are not specific for the detection of adducts formed by acyl glucuronides and may also be able to detect adducts formed by other reactive metabolites, including oxidative metabolites, sulphate conjugates, acyl-CoA conjugates and glutathione conjugates. Indeed, a recent study investigating the *in vivo* metabolic activation of 2-phenylpropionic acid in rats, demonstrated a significant contribution of both 2-phenylpropionyl-glucuronide and 2-phenylpropionyl-CoA to the generation of covalently bound adducts with liver proteins [91]. Thus, the role of acyl glucuronide metabolites in the *in vivo* formation of protein adducts by carboxylic acid drugs has largely been inferred based on comparisons with the protein adducts known to be formed by acyl glucuronides *in vitro* [84,104,105], or by establishing correlations between the extent of *in vivo* adduct formation with systemic/tissue exposure to the acyl glucuronide metabolite [68,79,82,84,104,105,107,108]. Interestingly, reports that the *in vivo* formation of diclofenac-protein adducts in rat liver are significantly reduced in Mrp2-deficient TR- rats compared to control Wistars [122], and that intestinal protein adduct formation by diclofenac is significantly reduced in rats with exteriorised bile flow [121], strongly suggest a role for a Mrp2 substrate (e.g. glucuronide, sulfonyl or glutathione conjugate) as the electrophilic protein-damaging species.

The *in vivo* consequences of protein adduct formation by acyl glucuronides are still relatively unclear. Significant *in vivo* adduct formation has been described in liver and many carboxylic acid drugs, particularly NSAIDs, are hepatotoxic. However, a direct role for acyl glucuronide-mediated cytotoxicity in hepatic damage has not been established. For NSAIDs, glucuronidation actually appears to decrease hepatic cytotoxicity [123], which may rather involve CYP-mediated formation of reactive metabolites [86], formation of reactive acyl-CoA conjugates [91] or uncoupling of mitochondrial oxidative phosphorylation [124,125]. It is possible that efficient canalicular excretion (via Mrp2/MRP2) and/or transporter-mediated sinusoidal efflux (e.g. *via* Mrp3/MRP3) may help to limit *in vivo* intrahepatic damage by acyl glucuronides. At the same time, efficient canalicular efflux may generate high concentrations of acyl glucuronides in bile, potentially acting as a toxification pathway for the biliary tree and intestines, and several studies provide *in vivo* evidence for acyl glucuronide-mediated small intestinal damage by NSAIDs (see Section 6.3). In addition, there is also *in vivo* evidence for a role of acyl glucuronides in immune-mediated toxicity.

Since the early identification of protein adduct formation by acyl glucuronides there has been concern that these metabolites may be able to act as haptens, and may thus be able to initiate immunological reactions. This hypothesis was supported by several observations. Firstly, some patients receiving valproic acid were reported to have measurable levels of antibodies against valproate-albumin adducts, although the levels were very low and unlikely to be clinically significant [53].

In addition, albumin covalently modified by tolmetin glucuronide or diflunisal glucuronide was shown to be immunogenic in mice [126] and rats [127], respectively. However, the albumin adducts had been prepared *in vitro* with epitope densities much greater than those observed *in vivo*, and the animals had also been treated with adjuvant. Finally, work utilising murine hepatocytes that had been pre-cultured with diclofenac (i.e. contained diclofenac-protein adducts) and spleen cells from mice that had been immunised with diclofenac conjugated to keyhole limpet hemocyanin (KLH), suggested that hepatocyte protein adducts may be immunogenic and lead to antibody-mediated hepatocyte damage [128]. Kretz-Rommel and Boesterli [128] reported that co-culture of diclofenac-pretreated murine hepatocytes with spleen cells from diclofenac-KLH immunised mice resulted in significant lymphocyte proliferation and antibody-mediated cytotoxicity, which was not observed in co-cultures utilising hepatocytes pretreated with vehicle only, or lymphocytes from mice treated with KLH only. However, although the authors reported measurable levels of diclofenac-protein adducts in the diclofenac-pretreated hepatocytes, a direct link between diclofenac acyl glucuronide-mediated adduct formation and immune-mediated cytotoxicity was not established. More convincing evidence for the involvement of acyl glucuronides in immune-mediated reactions comes from studies of drug-induced immunocytopenias, where a direct role for 4′-OH-diclofenac acyl glucuronide has been demonstrated in acute immune haemolytic anaemia reported in patients treated with diclofenac [129]. Interestingly, in this instance a non-covalent interaction between the acyl glucuronide and membrane proteins on the red blood cells may underlie conformational changes or formation of combinatorial epitopes, which stimulate the immune reaction [129].

4.2. Hydroxamic acid *N-O*-linked glucuronides

In contrast to acyl glucuronides, the formation of covalently bound protein adducts by the glucuronide conjugates of hydroxamic acids is poorly documented. This is most likely due to the identification of highly reactive *N-O*-linked acetyl and sulphonyl conjugates of arylhydroxamic acids and the subsequent focus on the role of these metabolites in the carcinogenicity of arylamines. Nonetheless, the *N-O*-linked glucuronide conjugate of *N*-OH-2-acetylaminofluorene has been shown to react with the amino acids L-methionine and L-tryptophan in aqueous solutions at pH 7–13 [130]. The reaction with methionine was pH dependent resulting in formation of 3-methylmercapto-2-acetylaminofluorene, 3-methylmercapto-2-aminofluorene and homoserine [130]. The formation of 3-methylmercapto-2-aminofluorene was proposed to proceed *via* pH-dependent deacetylation of the hydroxamic acid glucuronide conjugate to the highly reactive electrophilic arylhydroxylamine *N-O*-glucuronide [130]. At the end of 22 h incubations (37°C) at pH 8 and 9, formation of 3-mercapto-2-aminofluorene accounted for 2.5 and 5.6%, respectively, of the initial *N*-OH-2-acetylaminofluorene *O*-glucuronide present, increasing to 20% during similar incubations at pH 13. In contrast, under the same conditions, the aglycone *N*-OH-2-acetylaminofluorene did not react with either methionine or tryptophan [130].

5. Glucuronidation-Dependent DNA Damage

5.1. Acyl glucuronides

Despite the documented reactivity of acyl glucuronides with protein nucleophiles, relatively little is known about their ability to covalently modify DNA, although it is likely that acyl glucuronides can react with the nucleophilic $-NH_2$ groups of guanine, adenine and cytosine bases. The glycation of DNA by monosaccharides is well established and, like the glycation of proteins by monosaccharides, results in the formation of AGEs [131,132]. Importantly, DNA damage by monosaccharides and subsequent AGE formation is mutagenic [131,132]. Thus, it is important to determine whether acyl glucuronides are also able to access and damage nuclear DNA.

The first evidence for acyl glucuronide-mediated DNA damage emerged from our laboratory, in studies demonstrating that direct exposure of single-stranded bacteriophage DNA to clofibric acid glucuronide and gemfibrozil glucuronide resulted in large declines in transfection efficiency in *E. coli*, but that exposure to the parent aglycones had no effect [133]. In the same studies, incubation of double-stranded pSP189 plasmid DNA with clofibric acid glucuronide or gemfibrozil glucuronide was shown to produce extensive strand nicking, as detected by agarose gel electrophoresis [133]. The DNA-damaging potency of the acyl glucuronide conjugates was an order of magnitude greater than that of the endogenous monosaccharides glucose-6-phosphate and glucuronic acid [43,133], consistent with the more rapid formation of AGEs by acyl glucuronide-glycated proteins compared to monosaccharide-glycated proteins [42].

The ability of acyl glucuronides to access and damage nuclear DNA *in situ* was subsequently demonstrated in mouse hepatocytes cultured with clofibric acid or probenecid in the absence and presence of the glucuronidation inhibitor borneol [43,134]. In these studies, both drugs caused concentration-dependent DNA damage. Significant DNA damage was detected at concentrations within the ranges that may be achieved clinically and was abolished by co-incubation with borneol. More recently, in our laboratory, the UGT-dependent bioactivation of clofibric acid, probenecid, benoxaprofen and bezafibrate was demonstrated in studies utilising the human embryonic kidney cell line (HEK293), which does not constitutively express UGTs. None of the drugs caused DNA damage in control untransfected cells, however, all four drugs caused DNA damage in HEK293 cells transfected with human UGT enzymes known to metabolise carboxylic acids [135]. A number of fibrate hypolipidaemic agents have also been shown to induce sister chromatid exchanges, chromosomal aberrations and micronuclei in glucuronidation-proficient rat hepatocytes [136]. Taken together, these studies demonstrate that intracellularly generated acyl glucuronides are able to access and damage nuclear DNA. Although, at present, the mechanisms of acyl glucuronide-mediated DNA damage are not well understood, glycation mechanisms may be involved, since DNA damage in hepatocytes is inhibited by the glycation inhibitor aminoguanine [43].

The clinical consequences of acyl glucuronide-mediated DNA damage are currently unknown, and further work is necessary to investigate whether acyl glucuronides cause DNA damage *in vivo*, or whether such damage is cytotoxic or mutagenic. The fibrate hypolipidaemic agents are known hepatocarcinogens in rodents [137] and clinical use of clofibric acid was associated with significant hepato-biliary toxicity, including malignancies [138,139]. However, fibrate hypolipidaemic agents are consistently classified as non-genotoxic and their significant hepatocarcinogenicity in rodents has been attributed primarily to their activity as PPARα agonists [140]. Similarly, although there is evidence that probenecid [141] and several NSAIDs [142] cause neoplastic changes *in vivo* in rodents, they are not considered genotoxic. However, it is possible that current genotoxicity testing is unsuitable for the detection of glucuronide-mediated genetic damage (see Section 5.3).

5.2. Hydroxamic acid *N-O*-linked glucuronides

The *N-O*-glucuronide conjugates of arylhydroxamic acids have also been shown to form adducts with nucleosides, nucleotides, DNA and RNA *in vitro* [130,143–145]. These include the *N-O*-glucuronide conjugates of *N*-OH-2-acetylaminofluorene, *N*-OH-4-acetylaminostilbene, *N*-OH-4-acetylaminobiphenyl and *N*-OH-2-acetylaminophenanthrene. Formation of adducts was pH dependent, increasing with increasing alkaline pH [143–145]. In reactions with nucleic acids the relative order of reactivity of the *N-O*-glucuronides was *N*-OH-2-acetylaminofluorene *O*-glucuronide > *N*-OH-4-acetylaminostilbene *O*-glucuronide > *N*-OH-4-acetylaminobiphenyl *O*-glucuronide > *N*-OH-2-acetylaminophenanthrene *O*-glucuronide [145]. In each case, the parent aglycones were not significantly reactive [130,143,145]. The *N*-OH-2-acetylaminofluorene *O*-glucuronide was shown to react with guanosine 5′-monophosphate (GMP) to form both 2-aminofluorene-GMP and 2-acetylaminofluorene-GMP adducts [130]. Formation of the 2-aminofluorene-GMP adduct is thought to proceed *via* base catalysed formation of *N*-OH-2-aminofluorene *O*-glucuronide [73], thus accounting for the increased reactivity of arylhydroxamic acid *N-O*-glucuronides at alkaline pH and for the increased yield of nucleic acid adducts containing the 2-aminofluorene versus the 2-acetylaminofluorene moiety [144].

Although arylamines form covalently bound DNA adducts *in vivo*, the contribution of arylhydroxamic acid *N-O*-glucuronides to *in vivo* adduct formation or the *in vivo* carcinogenicity of arylamines is unclear, primarily due to the formation of other highly reactive electrophilic metabolites by these compounds. Nonetheless, subcutaneous injection of 12.6 μmol of *N*-OH-2-acetylaminofluorene *O*-glucuronide (in 0.9% NaCl solution) to rats 3 times a week for 9 weeks was associated with a 50% incidence of tumours by 13 months, compared to a 94% incidence of tumours by 13 months in rats administered 12.6 μmol aglycone 3 times a week for 9 weeks [146].

5.3. Limitations of current genotoxicity testing

Pre-clinical genotoxicity testing during drug development typically consists of the *in vitro* Ames reverse mutational assay (with or without microsomal enzymes to simulate bioactivation) combined with other *in vitro* assays including chromosomal aberration assays using Chinese hamster ovary (CHO) cells, forward mutational assays in mouse lymphoma cells or chromosome analysis in lymphocytes. In addition, *in vivo* genotoxicity studies are also carried out, usually in a rodent species, investigating micronucleus formation in bone marrow cells, and gross histological evidence for neoplasms.

The majority of standard genotoxicity assessments utilise cells and tissues that are not known to possess significant glucuronidation capacity and that are not known to express conjugate uptake transporters. Thus, it is likely that the genotoxicity of glucuronide conjugates has been significantly underestimated, primarily as a result of not adequately taking into account the enzymatic requirements for intracellular generation of glucuronide conjugates or the transporter requirements for cellular uptake of pre-formed glucuronides. For example, the glucuronidation capacity of bacterial cells used in the Ames test, or of lymphocytes used for micronucleus and sister chromatic exchange assays, is likely to be minimal. Although S9 bioactivation systems are commonly employed to enhance the metabolic capacity of *in vitro* assays, the cofactors necessary for glucuronidation (UDP-glucuronic acid and detergents to activate membrane bound UGTs) are rarely included, and the intra-cellular uptake of the hydrophilic glucuronide conjugates has not been demonstrated. It is therefore not surprising that, despite forming reactive glucuronide conjugates, many carboxylic acid compounds are classified as non-genotoxic based on *in vitro* testing, even when testing is carried out directly with the glucuronide conjugate. For example, although fibrate hypolipidaemic agents are traditionally classified as non-genotoxic using conventional genotoxicity assays [140], they consistently cause DNA damage in glucuronidation-proficient cells [43,134–136].

One strategy to address such limitations has involved modification of the Ames test by construction of Salmonella strains that express phase 1 or 2 biotransformation enzymes [147]. However, development of recombinant Salmonella that express UGTs has proved challenging and to date only a single enzyme (UGT1A1) has been successfully expressed [148]. Models such as UGT-transfected HEK293 cell lines may also provide an alternate approach for reassessing the role of UGT enzymes in drug genotoxicity.

6. Glucuronidation-Dependent Translocation of Toxins and Carcinogens

Whilst the liver is the most important site of *in vivo* glucuronidation, significant UGT expression has also been demonstrated in gastrointestinal mucosa and kidneys [4,149]. In these organs there is also significant expression of Mrp2/MRP2, the rodent/human apical efflux transporter mediating the extrusion of glucuronide (and other) conjugates into bile, the gastrointestinal lumen and

urine [150]. Thus, UGTs and MRP2 proteins are thought to act in combination to limit systemic exposure to toxins *via* metabolic detoxification followed by rapid cellular extrusion. Hence, MRP2 limits the intracellular exposure of hepatocytes, enterocytes and renal proximal tubular cells to glucuronide conjugates, whether they are stable detoxification products or chemically reactive (Figure 6). However,

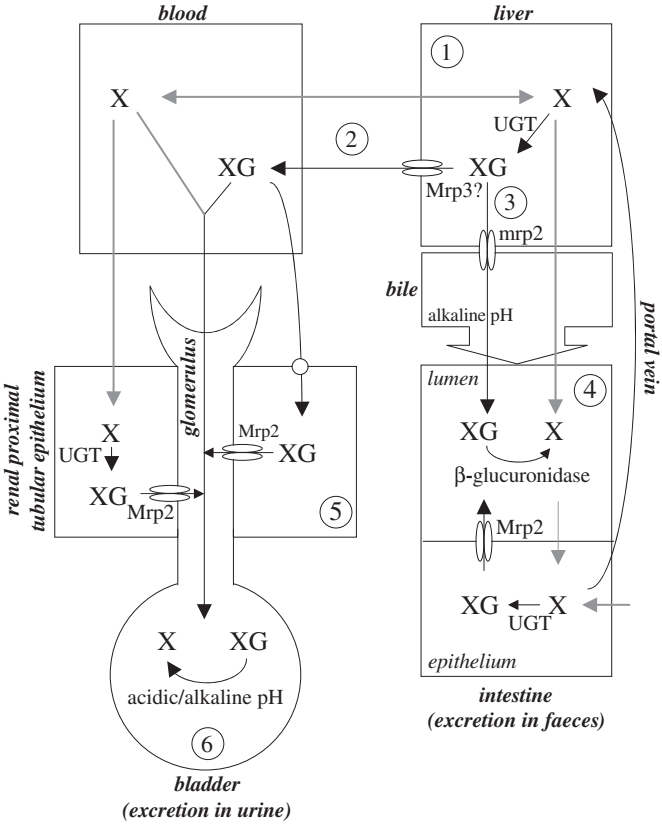

Figure 6 Formation and excretion of glucuronide conjugates in liver, gastrointestinal tract and urinary tract. ① Liver is the primary site of metabolism of endogenous and exogenous compounds (X) to glucuronide conjugates (XG), which are either transported by Mrp2/MRP2 across the canalicular membrane into bile ③, or by other active efflux transporters across the basolateral membrane into the circulation ②. Within bile glucuronide conjugates are exposed to an alkaline environment, facilitating non-enzymatic rearrangement and hydrolysis of reactive acyl glucuronides and hydroxamic acid *N*-*O*-glucuronides. The gastrointestinal tract ④ is exposed to glucuronide metabolites delivered by bile or following metabolism of the parent compound within the intestinal mucosa. Parent compound taken up into the small intestinal mucosa can be reabsorbed via the hepatic-portal vein. In the large intestine, *β*-glucuronidase can regenerate parent aglycone. Glucuronide conjugates present in the circulation ② can undergo glomerular filtration or active tubular secretion by the kidneys ⑤. Within renal proximal tubular cells parent drug can also be metabolised to glucuronide conjugates. Apical efflux transporters (such as Mrp2/MRP2) transport the conjugates into urine ⑥. Under mildly acidic or basic conditions reactive glucuronide conjugates can undergo non-enzymatic rearrangement or hydrolysis to reform the parent compound.

this efflux may result in significant exposure of the gall bladder and biliary tree, the intestinal lumen and the bladder to reactive glucuronide conjugates (Figure 6). In addition, the biliary tree, intestinal lumen and bladder may also be exposed to toxins and/or carcinogens that are locally re-generated by β-glucuronidase-catalysed hydrolysis of the biosynthetic 1-*O*-β glucuronides, or acid/base catalysed hydrolysis of the glucuronide conjugates of carboxylic acids, arylhydroxylamines or arylamines (Figure 6). Therefore, the combined actions of UGTs and MRP2 may have a number of toxicological consequences, including: (i) UGT-dependent intracellular detoxification followed by MRP2-dependent translocation of the conjugate to a distal site where the toxic aglycone is regenerated or (ii) UGT-dependent bioactivation followed by MRP2-mediated intracellular detoxification and translocation of the electrophilic reactive conjugate to distal sites where it may cause tissue damage or regenerate the parent aglycone. Given the important role of Mrp2/MRP2 in facilitating the excretion of compounds into the gastointestinal and urinary tracts, it is these organs that may be the most likely target-sites of glucuronidation-dependent toxicity. Indeed, exposure of the gastrointestinal tract to reactive acyl glucuronides is thought to be the cause of NSAID-induced enteropathy. In addition, a role for glucuronidation in the translocation of toxins to the gut lumen and bladder has been suggested for the intestinal toxicity of the cancer chemotherapy agent, irinotecan, as well as the bladder and colon cancer associated with environmental and food-derived arylamines. The contribution of biliary excretion to the delivery of toxins to the gastrointestinal tract, in general, was recently reviewed by Treinen-Moslen and Kanz [151].

6.1. Role of glucuronidation in the intestinal toxicity of irinotecan

Irinotecan (CPT11) is a camptothecin derived antineoplastic agent used for the treatment of metastatic carcinomas of the colon and rectum, small cell lung cancer and lymphoma. Irinotecan is a prodrug and is hydrolysed by carboxylesterases to form the active metabolite SN38 (Figure 7), a potent topoisomerase I inhibitor, that causes disruption of DNA replication, leading to irreversible double-strand DNA breaks and apoptosis. The major clinical dose limiting toxicities of irinotecan are severe diarrhoea and myelosuppression. Myelosuppression is consistent with the mechanisms of action of SN38. The diarrhoea observed in patients or rats administered irinotecan can be divided into acute early-onset diarrhoea, thought to be due to the anticholinesterase activity of CPT11, and severe delayed-onset diarrhoea (NCI-CTC grade 3–4 in 20–40% of patients) thought to be due to the mitotic inhibitory activity of SN38 [152]. SN38 is cleared primarily by glucuronidation (UGT1A1) and in both humans and rodents irinotecan, SN38 and SN38 glucuronide are excreted in bile and faeces [153–156]. In humans, approximately 62% of an i.v. irinotecan dose is excreted in faeces [154], and although the concentrations of SN38 glucuronide in bile are equal to or greater than those of SN38 [153,154] significantly more SN38 is excreted in faeces compared to SN38 glucuronide [154], consistent with hydrolysis of the conjugate

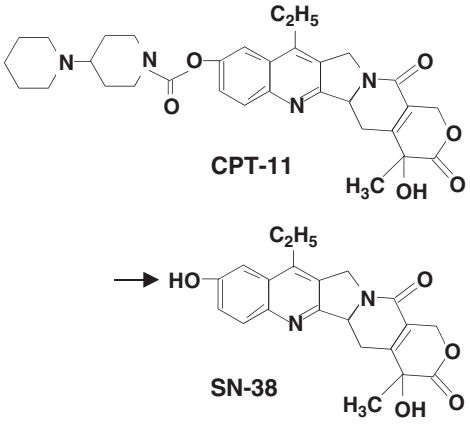

Figure 7 Chemical structures of irinotecan (CPT11) and its major metabolite, SN38. The arrow indicates the functional group conjugated to form SN38 glucuronide.

in the gut. Studies in rodents suggest that canalicular secretion of irinotecan is mediated by P-glycoprotein [155] and that of SN38 and SN38 glucuronide by Mrp2 [156]. Irinotecan is also metabolised by CYP3A4/5 to pharmacologically inactive oxidative metabolites [154].

Since irinotecan is administered clinically by i.v. infusion exposure of the gastrointestinal tract is most likely from excretion of irinotecan and its metabolites *via* bile, or direct uptake into mucosal cells from the circulation. Several studies suggest that although glucuronidation of SN38 acts as a systemic and hepatic detoxification pathway, its biliary excretion (*via* MRP2) and hydrolysis by β-glucuronidase, may be an important contributor to the exposure of the gastrointestinal tract to SN38 and subsequent delayed-type diarrhoea. For example, the severity of gastrointestinal damage in rats administered irinotecan i.v. for 4 days was greatest in the caecum, less in the ileum and colon and least in the jejunum, coinciding with the relative β-glucuronidase activity of the intestinal luminal contents [157]. In addition, diarrhoea and histological evidence of large intestine tissue damage was significantly reduced by antibiotic administration [157], which also decreased β-glucuronidase activity [157] and the concentrations of SN38 in faeces (by 80%), and increased the concentrations of SN38 glucuronide in faeces [157]. In a separate similar study, antibiotic treatment decreased the concentrations of SN38 in large intestine mucosal tissue (by 85%) and lumen (by approximately 90%), and significantly increased the concentrations of SN38 glucuronide in large intestine mucosal tissue and lumen [158]. In support of a direct effect of β-glucuronidase-mediated release of SN38 on diarrhoea and large bowel damage, antibiotic treatment had no effect on the tissue and luminal concentrations of irinotecan, SN38 or SN38 glucuronide in the small intestine where β-glucuronidase activity was low [158]. Similar results have been reported in a small clinical study with the aminoglycoside neomycin, an antibiotic with poor oral absorption. Neomycin significantly decreased the incidence and severity of delayed-onset diarrhoea in patients with colorectal cancer, without

affecting the plasma pharmacokinetics of irinotecan or SN38, consistent with an effect on β-glucuronidase activity in the large intestine, a poor site for drug absorption [159].

6.2. Role of glucuronidation in arylamine-induced bladder and colon cancers

Although, as discussed previously, the formation of reactive electrophilic hydroxamic acid *N*-*O*-glucuronides by arylamines may directly contribute to their carcinogenicity, arylamines and arylhydroxylamines can also form N-linked glucuronide conjugates that have not been shown to directly cause protein or DNA damage. Thus, *N*-glucuronidation of arylamines and arylhydroxylamines is considered a detoxification process that reduces the amount of aglycone available for *N*-oxidation and formation of reactive electrophilic oxidative metabolites or *N*-*O*-glucuronide, *N*-*O*-sulphonyl or *N*-*O*-acetyl conjugates (Figure 5). Although relatively stable at physiological pH, the N-linked glucuronides of arylamines and arylhyroxylamines become increasingly unstable at acidic pH (Table 1) [75,76]. The *N*-glucuronides of arylamines and arylhydroxylamines are excreted in urine, which is often mildly acidic, and are hydrolysed to re-form the parent aglycone [160], which can further react non-enzymatically to form highly reactive arylnitrenium ions and/or undergo further bioactivation within uroepithelial cells to DNA damaging ultimate carcinogens [161,162].

Kadlubar et al. [160] demonstrated the *in vitro* pH-dependent hydrolysis of *N*-hydroxy-2-naphthylamine *N*-glucuronide, *N*-hydroxy-1-naphthylamine *N*-glucuronide and *N*-hydroxy-4-aminobiphenyl *N*-glucuronide, as well as subsequent covalent binding with nucleic acids. At physiological pH, the conjugates were stable for up to 3 h incubation and no significant covalent binding to DNA was observed for up to 6 h incubation. In contrast, all three conjugates underwent extensive hydrolysis (90%) over 3 h at pH 5.0 and significant formation of adducts with nucleic acids was demonstrated, which continued to increase even after the conjugates had completely hydrolysed, suggesting formation of adducts by the parent arylhydroxylamines and not the glucuronides *per se* [160]. The *in vivo* role of *N*-glucuronides in the exposure of the bladder to carcinogenic arylamines is also supported by studies demonstrating an inverse

Table 1 Half-lives of *N*-linked glucuronide conjugates of arylamines and arylhydroxylamines in buffered aqueous solutions [76]

	pH			
	5.3	5.5	6.5	7.4
Benzidine *N*-glucuronide	5 min			1.7 h
N-acetylbenzine *N*′-glucuronide	4 min	7.5 min		2.3 h
N′-OH-*N*-acetylbenzidine *N*′-glucuronide		3.5 h	14 h	> 24 h

relationship between urine pH and the concentrations of unconjugated arylamines and arylhydroxylamines in urine (in rats and humans) [163,164] and the amount of DNA adduct in exfoliated bladder cells (in humans) [164]. However, since urine pH can affect the tubular reabsorption of weak acids and bases, an effect of acidic pH on the *in vivo* reabsorption of arylamines and arylhydroxylamines cannot be discounted.

A similar role has been postulated for the *N*-glucuronides of the food-derived heterocyclic aromatic amine carcinogens such as PhIP (2-amino-1-ethyl-6-phenylimidazo[4,5-b]pyridine), MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]-quinoxaline) and IQ (2-amino-3-methyl-3*H*-imidazo[4,5-f]quinoline). These compounds are formed during the cooking of meats at high temperatures and undergo bioactivation reactions similar to arylamines, involving hepatic *N*-hydroxylation followed by formation of electrophilic oxidative metabolites or electrophilic *N*-*O*-acetylated metabolites (Figure 8). Hepatic *N*-glucuronidation directly competes with bioactivation of the *N*-hydroxy metabolite, and is thought to be part of the metabolic detoxification pathways for heterocyclic aromatic amines [165–168]. Of the food-derived heterocyclic aromatic amines PhIP causes colon cancers in both humans and rats. *In vivo* studies using Wistar and Mrp2-deficient TR- rats have demonstrated the significant involvement of Mrp2 in the

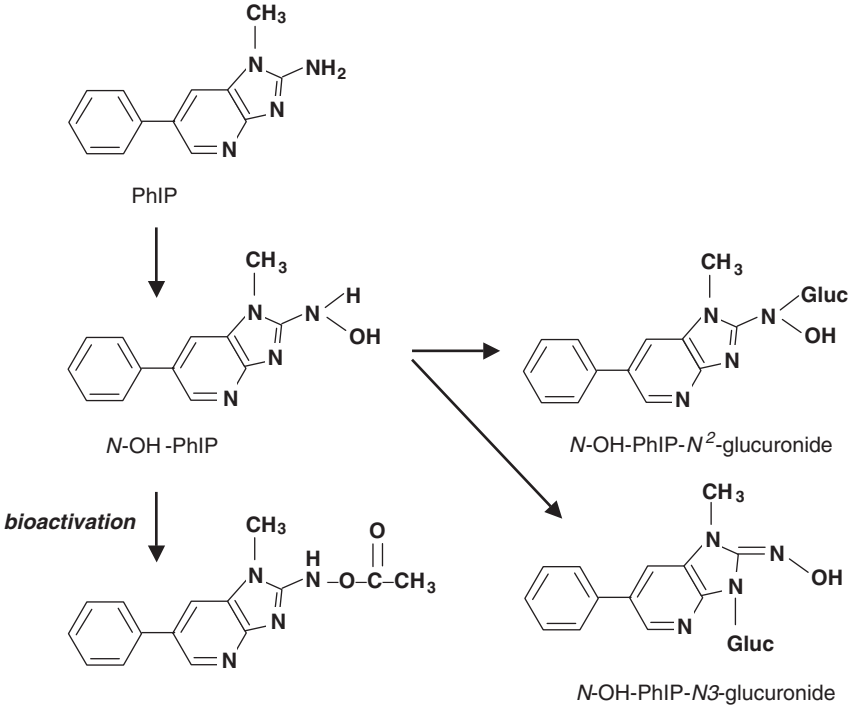

Figure 8 Major metabolic pathways involved in the bioactivation of PhIP and the detoxification of *N*-OH-PhIP.

biliary excretion of 4′-OH-PhIP glucuronide, *N*-OH-PhIP N^2-glucuronide and *N*-OH-PhIP N^3-glucuronide, with 90–100% loss of biliary excretion of these conjugates in TR- compared to Wistar rats [169]. To a lesser extent Mrp2 may also be involved in the hepatobiliary excretion of PhIP and 4′-OH-PhIP, since their biliary excretion was also significantly decreased in TR- compared to Wistar rats [169]. The role of glucuronidation as a means of transporting potential carcinogens to the gut was demonstrated by a recent *in vivo* study in Wistar and UGT1A-deficient Gunn rats administered an oral dose of ^{14}C-PhiP [170]. In this study, Gunn rats exhibited significantly reduced glucuronidation of PhIP and its metabolites as well as significantly reduced formation of DNA adducts in colon homogenates, compared to Wistar rats. In contrast, glucuronidation of *N*-OH-PhIP functions as a hepatic detoxification pathway, limiting DNA adduct formation and unscheduled DNA synthesis [171] in cultured primary rat hepatocytes.

6.3. Role of glucuronidation in the intestinal toxicity of NSAIDs

Gastrointestinal toxicity is a well-characterised side effect of NSAIDs. It comprises both gastroduodenal injury due to inhibition of cyclooxygenase (COX) activity, and also small intestinal injury (or enteropathy), that is not thought to be due to COX inhibition [172]. Several observations suggest that the agent producing small intestinal injury is delivered to its site of toxicity *via* bile, since in the rat model NSAIDs that are not subject to enterohepatic recirculation appear not to cause intestinal damage [173]; bile duct ligation, or cannulation and externalisation prevents intestinal damage [174,175]; and intestinal damage caused by diclofenac is practically abolished in TR- rats which do not express the Mrp2 canalicular transporter [122]. An association has also been proposed between NSAID biliary excretion and intestinal toxicity in humans [176]. Evidence for a direct role of NSAID acyl glucuronide conjugates in enteropathy comes mainly from studies in rats treated with diclofenac. In these studies, diclofenac acyl glucuronide is implicated as the intestinal toxin based on the observations that oral administration of diclofenac produces significantly less injury in TR- rats compared to control Wistars [177]; diclofenac acyl glucuronide is an Mrp2 substrate [122]; and induction of hepatic glucuronidation significantly increases the severity of intestinal damage in control Wistar rats [177]. There is also evidence that intestinal injury in rats may be related to formation of intestinal protein adducts by NSAIDs such as diclofenac, since intestinal diclofenac-protein adduct intensity and localisation mirrors ulcer incidence and localisation, and adduct formation appears to precede ulceration [174]. Adduct formation would not require uptake of the reactive conjugates into the intestinal epithelium, but could involve membrane proteins exposed to the extracellular lumen. In addition, the formation of β-glucuronidase-resistant rearrangement isomers of acyl glucuronides (as may occur during storage of bile in the gall bladder) has been proposed to facilitate transport through the small intestine and to the colon in rats [66] and, as discussed in Section 4.1, may also facilitate protein adduct formation.

7. SUMMARY

Due to their polarity and hydrophilicity, glucuronide conjugates do not readily diffuse across biological membranes, and their uptake into, and efflux out of, cells requires the action of membrane transporters. This fundamental property of glucuronide conjugates, coupled with Mrp2/MRP2-mediated cellular efflux, underpins the role of glucuronidation in facilitating the excretion of endogenous and exogenous compounds, thus completing the detoxification and deactivation cycle. However, it has also complicated our understanding of the *in vivo* and *in vitro* effects of pharmacologically active or reactive glucuronides, since exogenously preformed glucuronide conjugates are often tested for *in vitro* genotoxicity or cytotoxicity in cell systems that may lack appropriate uptake transporters, or are administered *in vivo* without taking into account their significantly different distribution compared to intra-cellularly generated metabolites. There are now numerous examples of glucuronide metabolites that retain pharmacological activity, that are bioactivation products, or that facilitate the transport of toxins to the gut or urinary tract. Our growing appreciation and understanding of the inter-relationship between UGT-catalysed metabolism and transporter-mediated cellular uptake and efflux will be essential to further assessing the contribution of glucuronidation to *in vivo* toxicity in humans and animals.

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CHAPTER 4

Allergic Contact Dermatitis — A Common Skin Disease Caused by Allergic Reactions to Chemicals in Our Environment

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1. Introduction

Allergic contact dermatitis (ACD) is the clinical result of skin contact with chemicals to which an individual is sensitized. A wide range of chemicals is known to cause skin sensitization after prolonged or repeated contact and ACD is an important occupational and consumer health problem. Within the western world 15–20% of the population are allergic to one or more chemicals [1]. In Germany, the "Berufsgenossenschaften", a health assurance system for the treatment and prevention of occupational diseases spends approximately 3 billion € per year for the treatment and prevention of ACD. Contact allergy can be diagnosed in dermatitis patients by patch testing using a standard tray of the about 30 most frequent contact allergens [2]. The most common causes of reactions to these standard allergens are metal salts (especially nickel), fragrance compounds and preservatives (Table 1). In certain clinics patch testing also is performed with the patient's own specific compounds or products to detect the offending agent(s). The technique of patch testing is presented in Figure 1. A positive patch test reaction in a patient allergic to colophony is shown in Figure 2.

The present review is focused on work that explores the chemistry and metabolism of skin sensitizers. The review also includes some dermatological aspects on the topic showing that contact with skin sensitizers is an important problem from both a society point of view and from an individual perspective. Clinically relevant examples illustrate common hapten–protein interactions. An increasing number of structure activity relationship (SAR) and quantitative structure activity relationship (QSAR) studies of potential relationships between physicochemical properties and contact allergenic effect have been published. It is however beyond the scope of this article to make an exhaustive review on these aspects.

Table 1 Frequency of positive reactions as determined by patch test results for the ten most common contact allergens or materials in the standard series at Department of Dermatology, Sahlgrenska University Hospital, Gothenburg, Sweden, 2005

Allergens	Total (%)	Men (%)	Women (%)
Nickel	18.7	2.9	26.1
Fragrance mix	10.7	8.1	12.0
Balsam of Peru[a]	7.8	8.1	7.6
Cobalt	5.0	1.2	6.8
Colophony	4.8	4.0	5.2
Chromate	3.9	4.0	3.8
MCI/MI[b]	2.6	1.7	3.0
Lichen mix	2.2	1.2	2.7
Neomycin	2.0	0.6	2.7
Formaldehyde	2.0	0	3.0

[a]Myroxylon pereirae.
[b]A mixture of 5-chloro-2-methyl-4-isotiazolin-3-one and 2-methyl-4-isothiazolin-3-one.

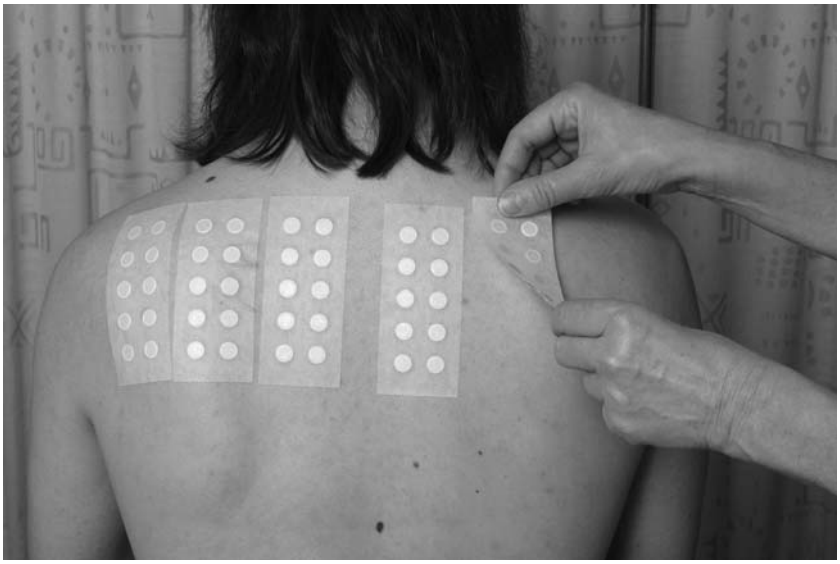


Figure 1 Patch test technique. A standard series of the most frequent skin sensitizers is applied on the back of a patient with suspected ACD. The sensitizers are mixed in suitable vehicles, most often petrolatum, to non-irritating and non-sensitizing concentrations. The patches remain on the skin for 48 h after which they are removed. On days 4 and 7 after the application of the test substances, the back of the patient is scrutinized to detect possible eczematous reactions on the spots for application. A small eczematous reaction shows that the patient is allergic to the compound applied on that spot.

2. Hand Eczema

The hands are most exposed to chemicals both at work and in daily life and thus most affected by contact dermatitis. Extensive epidemiological studies of hand eczema have been performed in Sweden in the last 10 years showing a prevalence of 9–10% in the normal populations of the two largest cities, Stockholm and Gothenburg [3,4]. It has also been shown that hand eczema affects the health-related quality of life [5].

Hand eczema is a disease of multifactorial genesis. The Swedish studies showed that a history of childhood eczema including atopic dermatitis, widespread hand dermatitis and an onset before the age 20 all influenced the prognosis negatively [6]. Contact allergy to standard allergens also influenced the prognosis negatively, but this is only a general estimation since an evaluation of the role of contact allergy in the long-term prognosis of hand eczema would have required information on contact allergen exposure in occupational and daily life [6].

Hand eczema can be caused by contact with irritants or allergens or by a combination of both. It is not possible to distinguish eczema of allergic origin from eczema caused by irritants by visual scoring or by histopathological investigations.

Figure 2 Positive patch test reaction to colophony.

Thus, not all cases of ACD can be diagnosed, since we are unable to find and test all chemicals with which every person comes in contact. Therefore, patch testing with standard trays is not sufficient but a correct diagnosis might include testing with further occupationally relevant allergens that are selected as a result of a carefully taken history. A combined exposure to irritants and allergens can increase the risk of ACD since the irritants damage the skin barrier, which increases the possibility for other chemicals to penetrate into the skin [7]. A synergistic effect of combined exposure to the contact allergen $NiCl_2$ (Ni^{2+}) and the commonly used model surfactant irritant sodium dodecylsulphate (SDS) was observed in the inflammatory response in nickel-sensitized individuals [8].

3. Mechanisms of Contact Allergy

3.1. Induction phase

Contact allergy is regarded as a type of delayed hypersensitivity (type IV hypersensitivity), which is mediated by allergen-specific T-cells [9]. To develop

ACD a person must be sensitized by skin contact with chemicals of a molecular weight below 1,000, which can penetrate into the viable epidermis. The chemical itself is too small to cause an immunogenic reaction, therefore its binding to a protein or peptide is a prerequisite to form hapten–protein complexes big enough to prime a cutaneous immune response. Reactions are considered to occur with different proteins, e.g., soluble proteins, or binding to proteins on the surface of the Langerhans cells (LC), which are the professional antigens presenting dendritic cells (DC) present in epidermis. The activation of the LC under the influence of tumor necrosis factor-α (TNF-α) results in a decreased expression of E-cadherine which fixes the LC to the keratinocytes resulting in a migration of the LC *via* the afferent lymph vessels to the draining lymph nodes where they can interact with and present the antigens to naive T-cells. These interactions result in the formation of antigen-specific effector and memory T-cells, which thereafter circulate in the blood and lymph vessels. On renewed contact with the same chemical these cells are recruited to the site of contact (Figure 3).

On their way to the lymph nodes the LC will mature and are no longer able to take up new haptens or hapten complexes but instead the hapten–protein complexes already encountered are processed. Mature LC have an increased ability to present the formed antigens on the major histocompatibility complexes (MHC) class I and II present on the surface of LC. Processing and subsequent binding to the surface of antigen presenting cells seem to be quite different among different compounds such as trinitrophenol, Ni ions or penicillin [10,11]. There are indications that antigen formation can take place by the direct interaction of Ni ions to the peptides of MHC class II [12–14]. Studies with Ni-reactive T-cells derived from Ni-allergic individuals preferentially expressed distinct T-cell receptor-Variable β (TCR-Vβ) chains with a significant overexpression of TCR-Vβ17(+) T-cells and their frequency correlated significantly with the *in vitro* reactivity of peripheral blood mononuclear cells (PBMC) to $NiSO_4$. In addition, the cutaneous infiltrate of Ni-induced patch test reactions consisted primarily of Vβ17(+) T-cells [15].

3.2. Elicitation phase

The circulating level of specific T-cells is usually below one per million cells in a non-sensitized individual, but after sensitization the level of specific effector cells can increase to one per thousand in the blood. If the individual does not come in contact with the same allergen again the frequency will gradually decrease but never return to the primary low level. When a person is sensitized to a specific compound a new contact with the same compound starts an immunological reaction resulting in ACD within a couple of days (Figure 3). In this case, memory T-cells will be recruited to epidermis, and interactions between T-cells and LC, and also with antigen-presenting keratinocytes, can take place directly in epidermis starting the inflammatory process (Figure 3). More effector cells are formed and recruited by the migration of antigen-presenting LC to the lymph nodes. Although the effector T-cells have an increased capability to escape from the vessels to the skin it will take hours or days to reach the full clinical picture of an

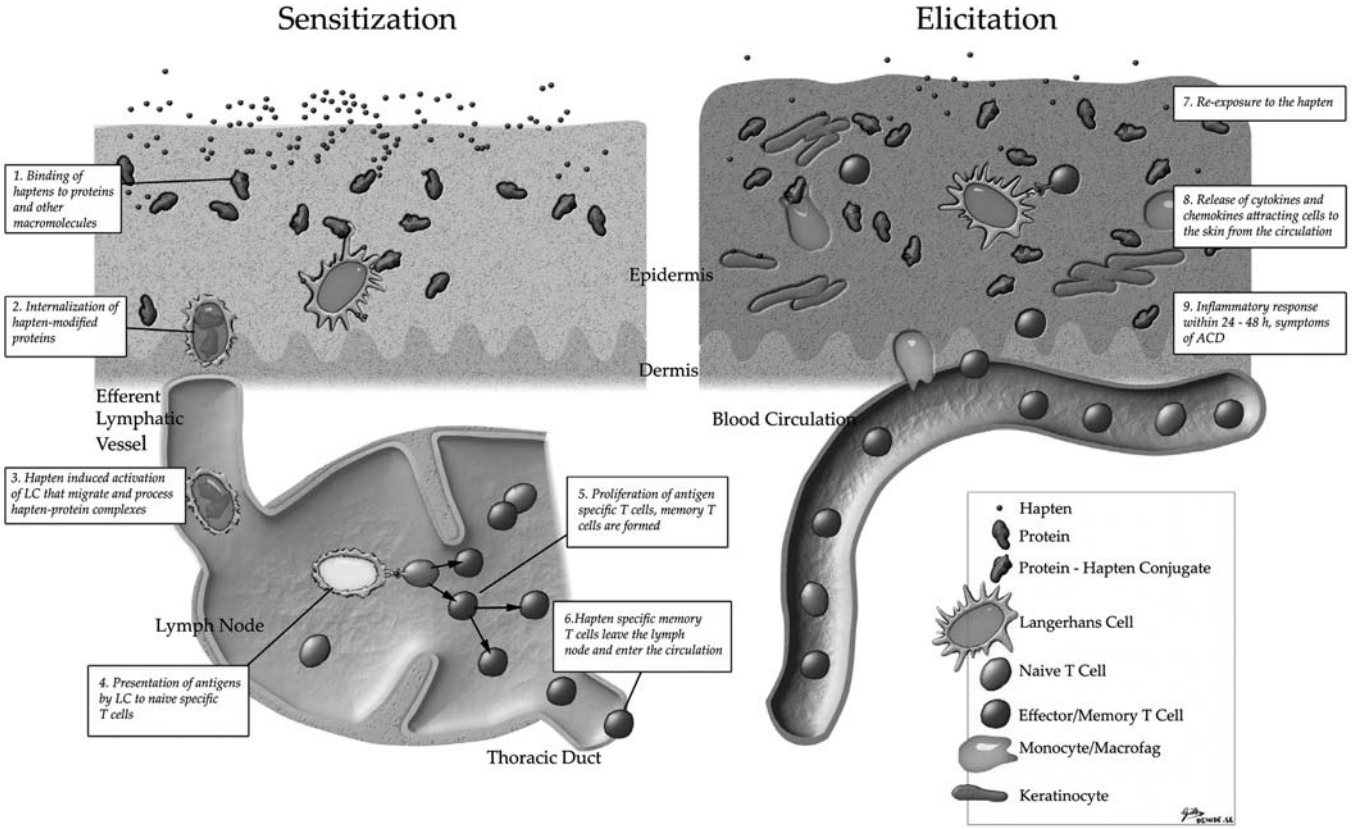


Figure 3 Mechanisms for induction of contact allergy and elicitation of ACD.

inflammation in the skin. Variations in the time course can show individual variations but also depend on the individual nature of the antigen (and the chemical). Thus, in clinical practice it is nowadays recommended that evaluation of possible skin reactions is performed 24 or 48 h as well as 72 or 96 h after application of the patch tests and also about a week after application [2].

3.3. Cytokines and chemokines involved in ACD

TNF-α and interleukin-1β (IL-1β) are the epidermal cytokines of mandatory importance for the migration of LC when these are activated by hapten–protein complexes [16]. Binding of TNF-α and IL-1β on their receptors expressed on LC is followed by a decreased expression of E-cadherine, which allows dissociation from encompassing keratinocytes [17,18]. Furthermore, TNF-α and IL-1β cause down-regulation of expression of chemokine receptors CCR1 and CCR5, that confer responsiveness to chemokines such as CCL3 (MIP-1α, macrophage inflammatory protein) and CCL5 (RANTES, regulated upon activation normal T-cell expressed and secreted) [19] and induce the expression of CCR7 and several adhesive molecules like CD44 or CD54 [20]. The ligands of CCR7 are CCL19 (MIP-3β) and CCL21 (SLC, secondary lymphoid tissue chemokine), which participate in the migration of DC from the skin to the regional lymph nodes and are crucial during the sensitization phase [21]. Additionally, it has been demonstrated that IL-18 induces LC migration in a TNF-α and IL-1β-dependent way [22].

The activation of T-lymphocytes is an important qualitative aspect of immune response to skin sensitizing chemicals, and the differential development of functional subpopulations of T-lymphocytes is central. Two major T helper (Th) subsets are described: Th1 and Th2 cells. IL-12 and interferon-γ (IFN-γ) play critical roles in promoting Th1 responses and IL-4 and IL-13 are important for induction of Th2 responses [23]. Th1 cells produce IL-2 and IFN-γ. They also express CXCR3, the receptor for CXCL10 (IP-10, interferon-induced protein), CXCL9 (MIG, monokine-induced by IFN-γ) and CXCL11 (I-TAC, interferon-induced T-cell α-chemoattractant) as well as CCR5, the receptor for CCL4 (MIP-1β) and CCL5. Th2 cells release IL-4, IL-5, IL-9, IL-10 and IL-13 and express CCR3, the receptor for CCL11 (eotaxin), CCR4, the receptor for CCL22 (MDC, macrophage-derived chemokine) and CCR8, the receptor for CCL-1 (I-309) [24,25]. T cytotoxic cells (CD8) display Tc1 and Tc2 phenotypes with cytokine production similar to Th1 and Th2 cells, respectively [26].

The recruitment of T-cells in the skin is regulated by the expression of appropriate homing receptors such as cutaneous lymphocyte-associated antigen (CLA) and chemokine receptors exposed on the T-cell surface or chemokines and cytokines released by activated resident skin cells [27]. The main stimulus for cytokine and chemokine release by keratinocytes is IFN-γ, frequently in combination with TNF-α and IL-17 [28]. CCL2 (MCP-1, monocyte chemotactic protein) is rapidly detectable after hapten challenge. Expression of CXCL10, CXCL9, CCL5, CCL17 (TARC, thymus- and activation-regulated chemokine) and CCL18 (PARC, pulmonary- and activation-regulated chemokine) starts 12 h after hapten application [29]. The trafficking of $CD4^+$ and $CD8^+$ T-lymphocytes is presumably

directed primarily by the CCR4/CCL17 (TARC) and CXCR3/CXCL10 (IP-10) axes, respectively [27].

The complex cytokine and chemokine production is responsible for the massive infiltration of leukocytes to the site of hapten challenge and amplifies the inflammatory response initiated by the hapten. Intervention of regulatory mechanisms to limit immoderate tissue damage is essential for maintaining the skin integrity. Specialized subsets of $CD4^+$ T-lymphocytes with regulatory functions have been described, such as Th3 cells, T regulatory (Tr) 1 cells and $CD4^+CD25^+$ cells [30]. Tr cells co-express Th1- and Th2-associated chemokine receptors with high levels of CCR8 and moderate amounts of CCR7 [31]. IL-10 has been shown to play a crucial role in the down-regulation of murine reactions to contact sensitizers. For instance, IL-10 injection before challenge totally abrogated the response [32]. IL-10 inhibits the maturation of DC including the IL-12 release, thereby diminishing their capacity to activate specific Th1 and Tc1 lymphocytes. The production of IL-10 is not restricted to T-cells but can be released locally by other cells like keratinocytes [33].

Because of the ability of T-cell clones to produce IFN-γ and additionally their ability to produce local passive cell transfer *in vivo*, the contact hypersensitivity appeared as mainly Th1 cytokine driven [34,35]. During the past years, it has become clear, however, that this exclusiveness might not reflect the physiologic situation. Ten years ago, the group of Asherson et al. [36] pointed out that many Th1 cell clones were unable to evoke contact hypersensitivity when administered intravenously (i.e., no systemic transfer was possible). However, treatment of the recipient mice with low doses of IL-4 before injection allowed a systemic transfer, whereas treatment of the mice with IL-4 monoclonal antibodies abrogated the systemic response. Therefore, it was postulated that IL-4 might play a critical role in the effector phase of contact allergy. Interestingly, in a recent clinical study [37] the large majority of biopsy specimens analyzed in this study displayed high expression levels ($>$5-fold median increase) of IL-4 and IL-13 (89.3 and 85.7%, respectively), whereas a more than 5-fold increase in IFN-γ mRNA levels was only detected in a limited number of ACD patients (26.8%). Also, mRNA levels of IL-31 were significantly increased in more patients (42.9% showed a more than 5-fold increase) [37]. Indeed, T-cell clones (TCC) with Th1-like cytokine pattern have been described being involved in ACD [38] but later also Th2-like cytokines have been observed for nickel- and PPD-specific TCC [39–41]. Recently, it was revealed that different metal ions (nickel, cobalt, chromium, palladium and gold) induce a mixed Th1- and Th2-type cytokine response in PBMC from sensitized individuals [42,43]. Using the same methods, a mixed cytokine pattern was also observed for the organic chemicals 5-chloro-2-methyl-4-isotiazolin-3-one, and 2-methyl-4-isothiazolin-3-one (MCI/MI) in PBMC from individuals sensitized to MCI/MI [44]. Kitagaki et al. [45] demonstrated that repeated application of 2,4,6-trinitrochlorobenzene (TNCB) caused a shift of the cutaneous cytokine pattern from Th1 to Th2. Furthermore, penicillin-specific TCC showed a shift from Th1-like cytokine profile to Th2-like cytokine profile depending on the allergen concentration [46]. The obtained

results emphasize that ACD is a very complex and dynamic process, which is influenced by numerous different factors.

4. Hapten–Protein Interactions

To act as a sensitizer a chemical must gain access to the viable epidermis (Figure 3). This requires that the chemical has the physicochemical properties necessary for passage across the horny layer (stratum corneum). Experimental studies have shown that the critical determinant of the effectiveness of sensitization is the amount per unit skin area of a compound [47]. Clinical experience and experimental studies have shown that the possibility for a chemical to cause contact allergy when entering viable epidermis depends on its reactivity. The basic hypothesis is that only protein-reactive compounds (or those compounds that can be activated by e.g., air oxidation or metabolically in the skin) are able to haptenate proteins and act as skin sensitizers, most often by the formation of covalent bindings in electrophilic–nucleophilic interactions [48,49]. This was a postulation that was first made by Landsteiner and Jacobs in 1935 [50,51]. Some investigators claim that a direct binding between a hapten and a protein is not necessary to start an immunologic reaction. Instead, a more unspecific interaction leading to so-called cryptic epitopes, i.e., self-proteins are changed in a way that they are recognized as foreign proteins by the immune system, is the major mechanism for skin sensitization. Studies with T-lymphocyte clones of individuals who were sensitized to small molecular weight compounds revealed that a processing of these haptens is not necessary to activate the T-lymphocyte [41,52]. This led to the p-i-concept (p, pharmacologic; i, immunologic), but one has to keep in mind that it has not been shown till yet that this is also the case in the induction phase. It is important to remember that nobody has been able to investigate the antigen formation in situ in the skin and therefore the studies performed so far only mirror what is believed to occur in real life. However, the increasing amount of studies using sophisticated analytical methods and SAR calculations show that the formation of covalent bonds between electrophilic haptens and nucleophilic sites in the skin proteins is a plausible explanation of the sensitizing effect seen for many clinically relevant chemicals [53]. The interaction with the amino acid residues varies depending on the chemical reactivity of the electrophile, e.g., for some compounds reactions with the primary amine in lysine residues dominate, while for others the major reactivity is seen with the thiol in cysteine. Also, other amino acids with heteroatoms, e.g., histidine, methionine and tyrosine, have been observed as nucleophilic moieties in reactions between peptides or proteins and various contact allergens.

The experimental findings do not exclude alternative routes of hapten–protein interactions. Regarding hydroperoxides that are identified as strong sensitizers in animal studies and in clinical investigations, the role of direct radical reactions forming covalent bonds between the hapten and the protein has been studied but

needs further investigation [54–56]. Furthermore, the formation of cryptic epitopes cannot be excluded.

4.1. Electrophilic–nucleophilic interactions

4.1.1. Michael addition

A majority of the strong sensitizers identified are so-called Michael acceptors. In experiments with peptides and proteins, it has been shown that the thiol group in cysteine reacts with the β-carbon in the electrophile when a double bond is conjugated to a carbonyl group in the hapten (Figure 4).

Sesquiterpene lactones with an *α-methylene-γ-butyrolactone* as the Michael acceptor in the hapten–protein interactions are the major skin sensitizers in plants from the Compositae (Asteraceae) family [57,58] (Figure 4). Exposure to Compositae plants is an important cause of ACD worldwide [59] and frequent reports are found in literature of occupational ACD, e.g., in gardeners and greenhouse workers due to contact with plants of this family [60]. Indian ragworth (Parthenium hysterophorus), a weed from the Compositae family has caused epidemic ACD [61]. A mix of three specific sesquiterpene lactones is used in the standard series for screening of ACD [62]. Since many different sesquiterpene lactones can cause ACD and we also find variations in structure between the lactones from the same species when growing in different geographic areas it is not surprising that this sesquiterpene lactone mix only detects approximately 30% of patients with allergy to plant lactones [62,63].

Other Michael acceptors are *α,β-unsaturated ketones* (Figure 4). On the basis of the reactivity of glutathione (GSH) with α,β-unsaturated ketones it has been demonstrated that monosubstitution at the β-position decreased the nucleophilic addition by approximately 1,000 times, while disubstitution reduced the relative rate of reaction by more than 1,00,000 times [64]. The same study also showed that the reactivity of allylic cyclic ketones was decreased compared to that of allylic non-cyclic ketones. One of the most widely used flavoring and fragrance substances is the α,β-unsaturated cyclic ketone carvone, which also has been shown to be a skin sensitizer [65]. Carvone is found in e.g., spearmint oil and also as a secondary oxidation product in air-oxidized limonene [65]. Clinical cases of ACD to carvone are described [66]. In an investigation including synthesis of model compounds of carvone, reactivity experiments with nucleophiles and peptides and sensitization studies in animals, it was demonstrated that only reactions as Michel acceptor caused skin sensitization and that both methylcarvone and dihydrocarvone were non-sensitizing [67] (Figure 5).

α,β-Unsaturated aldehydes are important sensitizers acting as Michael acceptors (Figure 4). Cinnamic aldehyde is the most well-known example. It is a common fragrance chemical included among the fragrance allergens in the standard series for screening of contact allergy among dermatitis patients. A QSAR model based on reactivity factors and lipophilicity has been developed for unsaturated aldehydes with a carbonyl function conjugated with one or more double bonds [68]. The sensitizing capacity of the aldehydes was determined experimentally according to

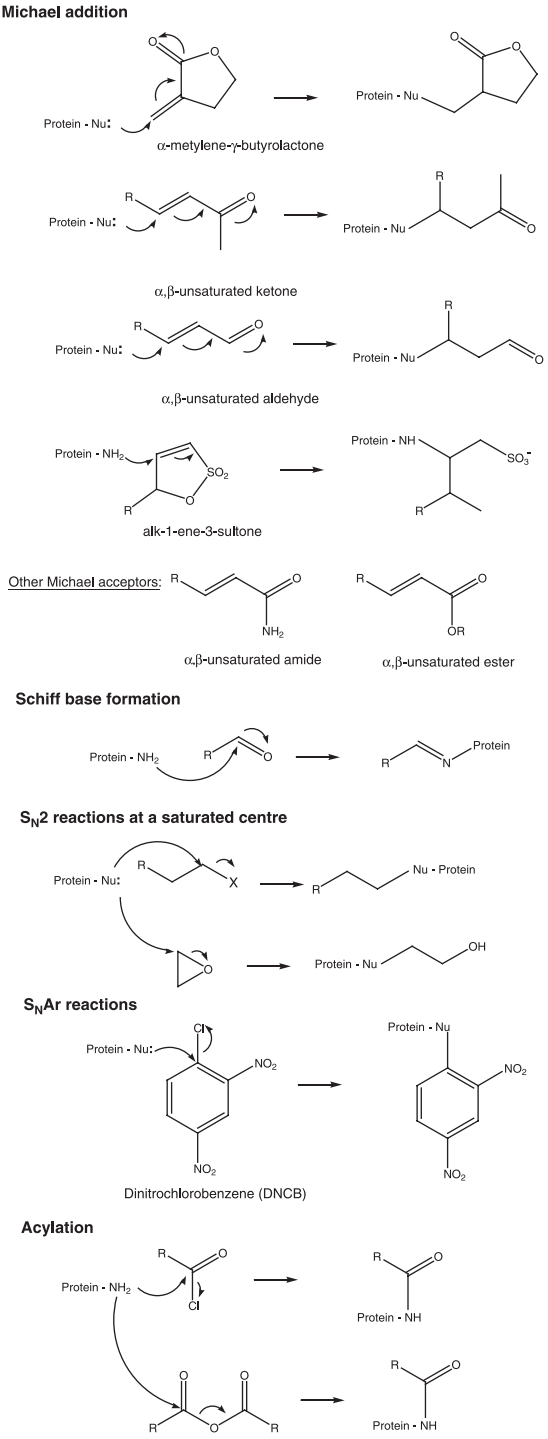


Figure 4 Proposed interactions between electrophilic haptens and nucleophilic sites in skin proteins forming covalent bonds.

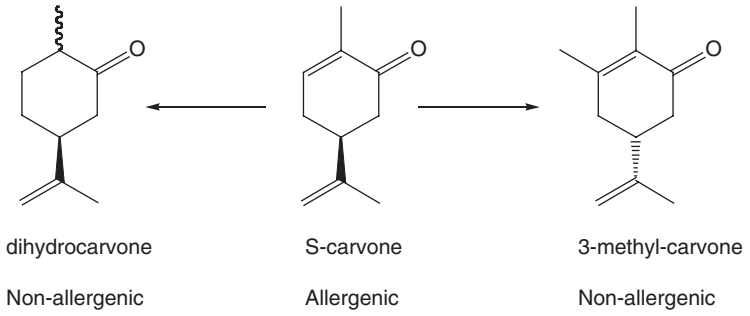


Figure 5 Carvone, an α,β-unsaturated ketone that was shown to act as a Michael acceptor in experimental studies. Hydrogenation of the α,β-double bond as well as introduction of a β-methyl group as a steric hindrance resulted in inactive compounds [67].

the local lymph node assay (LLNA) in mice [69]. For further description of the LLNA see Section 7.1.

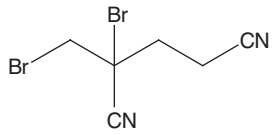
Michael addition has been shown to be important for the sensitizing capacity of *α,β-unsaturated sultones* (Figure 4). An outbreak of ACD in Scandinavia at the end of 1960s was caused by dishwashing liquids containing lauryl ethersulphate [70]. The cause of the dermatitis was traced to minute amounts of sultones as contaminants formed when lauryl ethersulphate was produced at high temperatures [71]. Especially, the α,β-unsaturated sultones were shown to be strong sensitizers in animal experiments [72]. Using ^{13}C-labelled hexa-1,3-diene sultone and hex-1-ene sultone as model compounds recent studies have shown that both saturated and unsaturated sultones react with tyrosine, but that only the unsaturated compounds are able also to act as a Michael acceptor in reactions with lysine [73,74].

4.1.2. Schiff base formation

Saturated aliphatic aldehydes are the most important chemicals in this group (Figure 4). The Schiff base formation in proteins takes place with the primary amines in lysine residues. The aldehydes in this group are less reactive and less sensitizing compared to the α,β-unsaturated aldehydes reacting as Michael acceptors. A QSAR equation has been developed also for the Schiff base aldehydes in the same way as described above for the Michael acceptor aldehydes [68,75]. A QSAR expression applicable across "the whole Schiff base reaction mechanistic applicable domain" was recently presented [76].

4.1.3. S_N2 reactions at a saturated center

Substitution reactions are another possible way for protein interactions of which the S_N2 reactions at a saturated center is the most important reaction, e.g., for alkyl halides (Figure 4). During the past decade, one alkylhalide, 1,2-dibromo-2, 4-dicyanobutane (methyldibromoglutaronitrile, MBDGN) (Figure 6) caused world wide outbreaks of ACD when used as preservative in cosmetics and toiletries which was observed as an increasing number of contact allergic reactions in patch test



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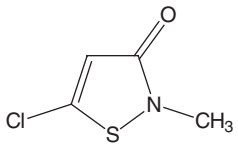

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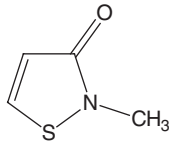


3

Figure 6 Efficient antimicrobial agents that have turned out to be potent contact allergens: Methyldibromoglutaronitrile, MBDGN (1,2-dibromo-2,4-dicyanobutane) 1; MCI (5-chloro-2-methyl-4-isotiazolin-3-one) 2 and MI (2-methyl-4-isothiazolin-3-one) 3 are used together in different commercial preservatives.

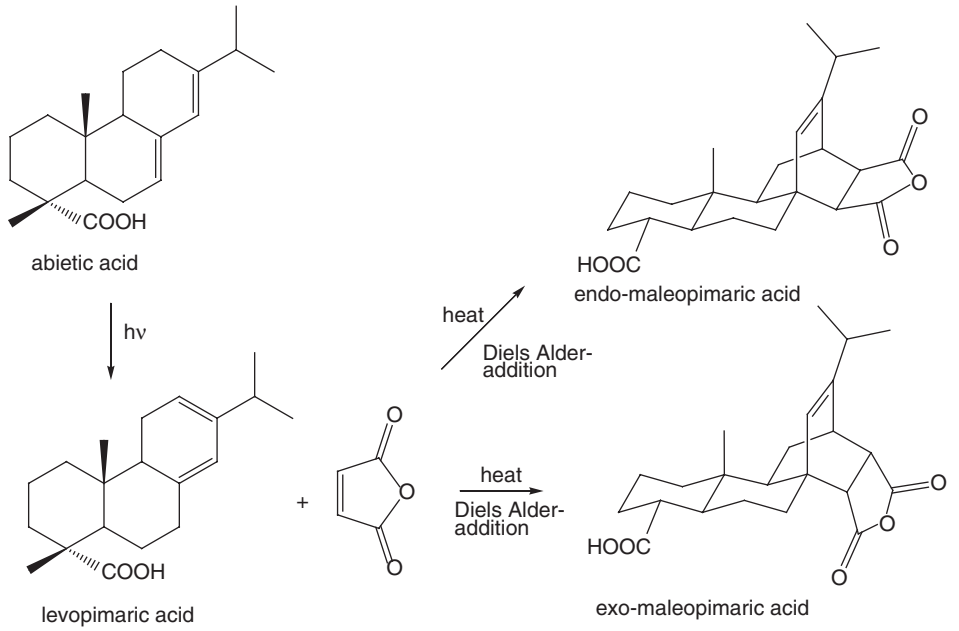
clinics [77]. As a result, this preservative was forbidden in all cosmetic products in March 2007 according to the European Union legislation.

4.1.4. S_NAr reactions

These reactions are mainly found with experimental sensitizers such as dinitrochlorobenzene (DNCB) (Figure 4). Interestingly, DNCB was used in the first investigation where the importance of an electrophilic–nucleophilic interaction between a compound and proteins in skin was postulated as an explanation to the sensitizing capacity of the same compound [50]. Since then, DNCB has been used in numerous experimental studies regarding contact allergens through the years. It has also been used for treatment of alopecia areata to cause inflammation in the scalp, which makes the hair grow again.

4.1.5. Acylation

Alifatic anhydrides, acid chlorides and isocyanates can act as acylating agents of amino acids with primary amines, specifically lysine residues, in proteins and form skin sensitizers (Figure 4). An example of a potent sensitizer is maleopimaric acid that is formed when rosin (colophony) is modified using maleic anhydride [78] or



Scheme 1 Maleopimaric acid is a potent contact allergen formed in the Diels–Alder reaction between levopimaric acid from colophony and maleic anhydride. Endo-maleopimaric acid is the major product obtained [78,79].

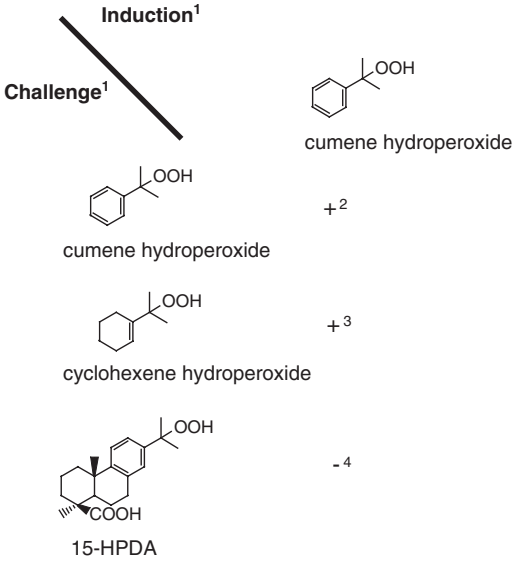
fumaric acid [79]. Diels–Alder adducts are formed between the dienophile (maleic anhydride or fumaric acid) and levopimaric acid, a suitable diene in rosin (Scheme 1). Cases of contact allergy to maleopimaric acid, which otherwise would have been undiagnosed, were detected when this compound was used for screening among dermatitis patients [80].

4.2. Radical mechanisms

The possibilities of a radical mechanism involved in the formation of antigens have attracted increased interest [54–56,81]. It has been shown that hydroperoxides are important haptens (see Section 5.1). Theoretically, structurally different hydroperoxides and peroxides could form the same oxidized and immunogenic protein since they are oxidizing agents and thus may oxidize functional groups in the proteins. However, no general cross-reactivity was observed in sensitization studies on different hydroperoxides in guinea pigs [82]. Cross-reactions between cumene hydroperoxide and cyclohexene hydroperoxide show that similarity in the overall structure and the way of antigen formation is needed (Table 2).

The activity of hydroperoxides as haptens could involve radical reactions either by forming covalent bonds with proteins by an inter-molecular radical reaction or by intra-molecular radical rearrangements to form epoxides that in turn can act as electrophilic haptens. Indications on both ways of reactions have been presented. In a cross-reactivity study in guinea pigs [54,83] on the major allergens in

Table 2 Cross reactivity studies regarding allergenic hydroperoxides [82]



[1] Sensitization studies performed in guinea pigs according to Freund's Complete Adjuvant Test
[2] Positive reactions in animals induced with cumene hydroperoxide when challenge tested with cumene hydroperoxide
[3] Positive reactions in animals induced with cumene hydroperoxide when challenge tested with cyclohexene hydroperoxide
[4] No reactions in animals induced with cumene hydroperoxide when challenge tested with 15-hydroperoxydehydrobietic acid (15-HPDA)

colophony, 15-hydroperoxyabietic acid (15-HPA), 15-hydroperoxydehydroabietic acid (15-HPDA) and epoxides of abietic acid, it was demonstrated that 15-HPA cross-reacted with both 15-HPDA and the epoxides (Table 3). A possible route for protein modification from carbon-centred radicals derived from an allergenic hydroperoxide was indicated in a study using electron paramagnetic resonance (EPR) spin trapping [84].

4.3. Metal binding

Some metal ions are important sensitizers in the population, especially nickel ions [2] (Table 1). In the case of metal ions, co-ordination bonds are formed between the ion and electron-rich atoms, mainly oxygen, nitrogen and sulphur (ligands). The sensitizing capacity of the metal depends on the number of ligands and the geometry of the co-ordination complexes [85].

5. Activation of Prohaptens

Chemicals that are not reactive themselves can be activated by autoxidation in contact with atmospheric oxygen or by skin metabolism. A third way, photo-activation will not be dealt with in the present review.

Table 3 Cross reactivity studies regarding allergenic oxidation products from colophony [83]

Challenge[1] \ Induction[1]	15-HPDA[2]	15-HPA[3]	α-epoxy-abietiate	β-epoxy-abietiate
15-HPDA[2]	+[4]	-[5]	nt[6]	nt
15-HPA[3]	+	+	+	+
α-epoxy-abietiate	nt	+	+	+
β-epoxy-abietiate	-	+	+	+

[1] Sensitization studies performed in guinea pigs according to Freund's Complete Adjuvant Test
[2] 15-Hydroperoxydehydrobietic acid Me-ester; [3] 15-Hydroperoxyabietic acid Me-ester (Isolated and identified as their methyl esters.)
[4] Positive reactions in animals induced with 15 HPDA when challenge tested with 15-HPDA
[5] No reactions in animals induced with 15HPA when challenge tested with 15-HPDA
[6] nt = not tested

5.1. Autoxidation

Autoxidation is induced by air-oxidation of organic molecules and the allylic hydrogen atoms are the most plausible targets, involving a hydrogen abstraction in a radical chain process. The free radical reaction is generated by exposure to light, heat or catalytic amounts of transition metals. Since many terpenes are unsaturated compounds with allylic hydrogen atoms available, they have been shown to autoxidize at handling and storage. The primary oxidation products formed at autoxidation are hydroperoxides, and terpene hydroperoxides have been identified as strong skin sensitizers. From hydroperoxides secondary oxidation products are formed due to oxidative decomposition (Figure 7).

Turpentine used for painting was a well-known cause of occupational ACD in the first half of the 20th century. Turpentine is obtained from pine trees and consists of monoterpenes. In the 1950s, it was proposed that the offending agents were hydroperoxides formed by autoxidation of Δ^3-carene (Figure 8), one of the monoterpenes in turpentine [86,87].

Since turpentine was soon replaced by synthetic solvents further research on its allergenic activity was not performed. However, the other part of the exudates from pine trees, colophony (rosin) has been of continuous importance within industry e.g., in paints, glues, printing inks and for paper sizing and also in products in daily life, e.g., as adhesive in plasters. Colophony is one of the most common causes of contact allergy in the standard series of patch test materials (Table 1). It has a complex chemical composition but in the middle of the 1980s it was demonstrated

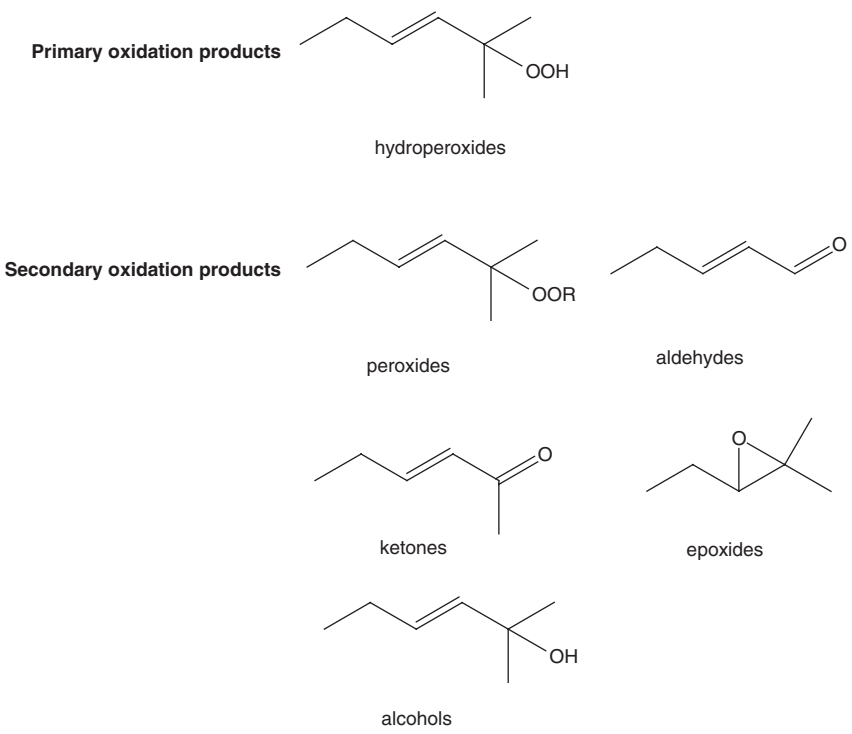


Figure 7 Primary and secondary oxidation products obtained from terpenes with allylic hydrogen atoms available for a free radical reaction with oxygen in air.

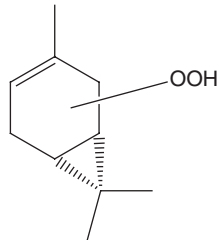


Figure 8 Hydroperoxides of Δ^3carene were identified as the major contact allergens in turpentine in the 1950s [86,87].

that highly sensitizing compounds are formed due to autoxidation of the major compounds (diterpenes) of colophony at air exposure. 15-HPA, the primary oxidation product of abietic acid, was identified as the major allergen in colophony [88]. However, other hydroperoxides and secondary oxidation products were also identified as skin sensitizers [89–91]. Some of the sensitizing oxidation products identified are shown in Table 3. An important finding was that the identified hydroperoxides were more stable than was expected based on general reports in literature and common knowledge among chemists. Thus, the hydroperoxides

could be isolated or synthesized, there sensitizing potential experimentally determined, and they could be used for patch testing in dermatitis patients. In clinical studies, about half of the patients with contact allergy to colophony react to 15-HPA [92,93].

Later research has shown that monoterpenes such as the most common fragrance terpenes, limonene and linalool, autoxidize and form oxidation products of which the hydroperoxides are the major sensitizers (Figure 7). At the end of the 1980s, limonene (citrus oil) was advertised as the environmentally friendly alternative to ordinary industrial solvents. However, very little was known about its toxicological effects at that time. Investigations showed that limonene autoxidizes at storage and handling and that autoxidized limonene is a frequent cause of contact allergy due to its use as a fragrance compound [65,94–99] (Figure 9).

Linalool from lavender oil is the most frequently used fragrance terpene. Thorough investigations with regard to chemistry, skin sensitizing effect and clinical importance have been performed on autoxidation and oxidative decomposition of linalool (Figure 10). These investigations show that linalool also forms allergenic oxidation products at air exposure, which are common sensitizers in dermatitis patients [100–102] (Figure 11).

Also, the autoxidation of caryophyllene from clove oil has been investigated [102,103]. Since the formation of allergenic compounds from fragrance terpenes

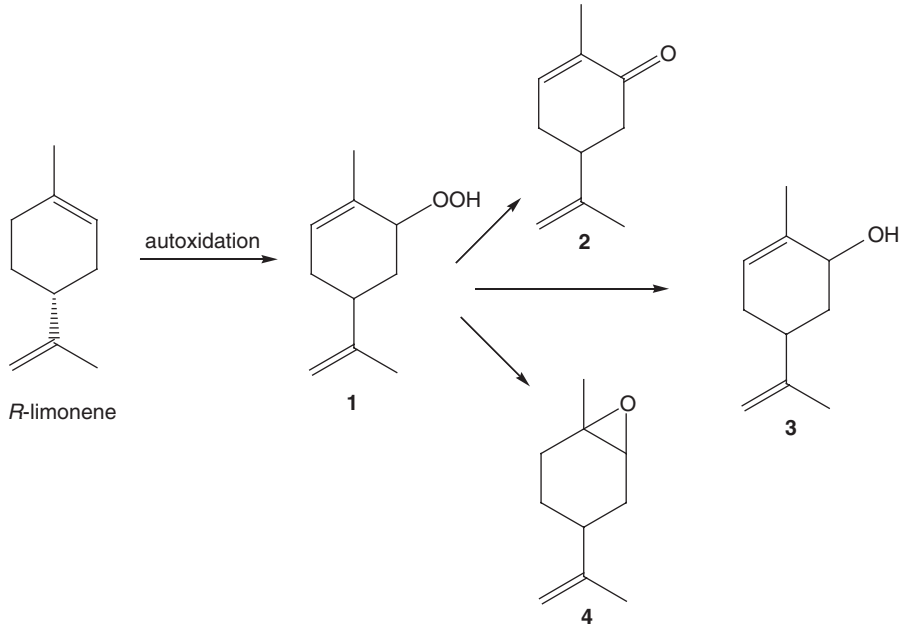


Figure 9 Primary and secondary oxidation products identified after autoxidation of *R*-limonene in room temperature. Limonene-2-hydroperoxide 1 is a strong sensitizer, carvone 2 is a moderate sensitizer, limonene-2-alcohol 3 is a non-sensitizer and limonene-1,2-epoxide 4 is a weak sensitizer according to guinea pig studies and LLNA studies in mice.

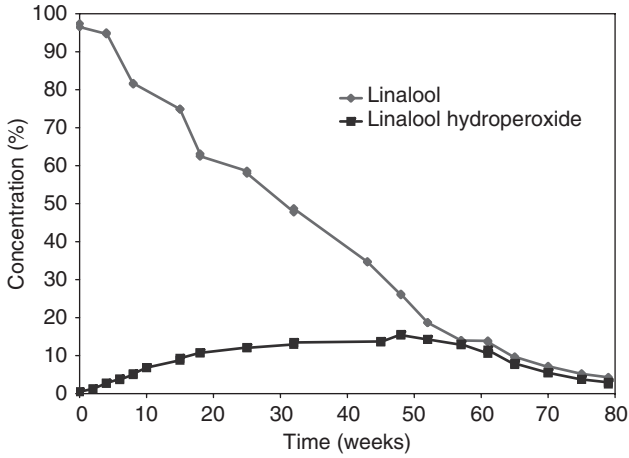


Figure 10 Oxidative degradation of linalool and formation of linalool hydroperoxides over time at air exposure in room temperature.

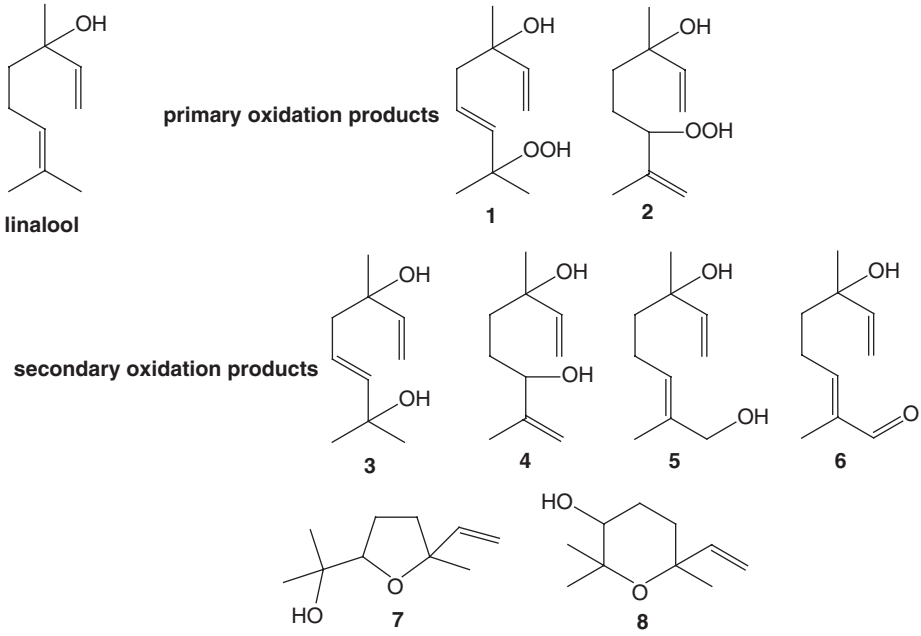


Figure 11 Primary and secondary oxidation products identified after autoxidation of linalool in room temperature. Linalool hydroperoxides 1 and 2 are strong sensitizers, linalool aldehyde 6 is a moderate sensitizer, while all other identified compounds are weak or non- sensitizers according to the LLNA in mice.

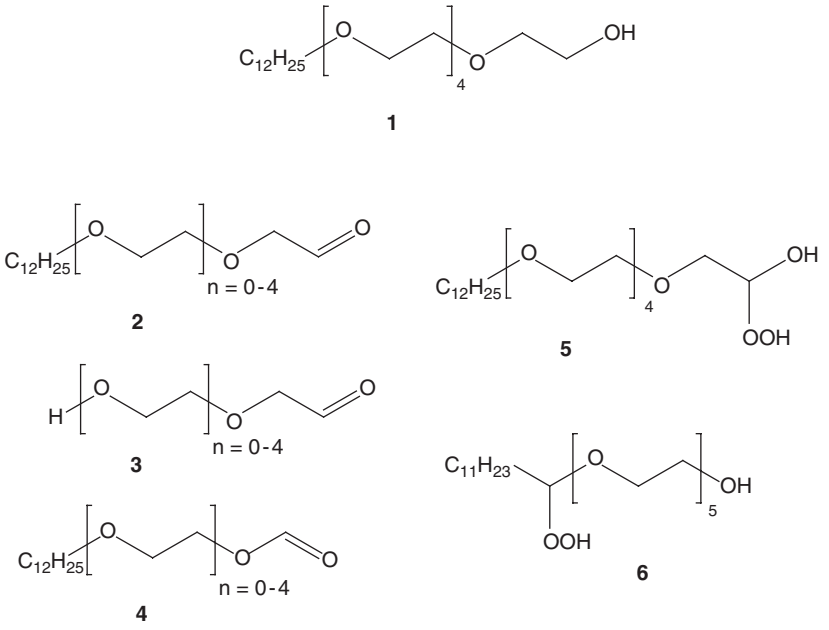


Figure 12 Primary and secondary oxidation products identified after autoxidation of penta-ethylene glycol mono *n*-dodecyl ether 1 in room temperature. The formates 4 were non-sensitizing, while the other investigated oxidation compounds were allergens according to guinea pig studies.

was not previously considered, these fragrance materials have been patch tested in their non-oxidized form resulting in very few positive reactions [104,105]. After the new findings screening was performed in thousands of patients at dermatology clinics in Europe. It was revealed that 2–3% of the tested patients reacted to the oxidized materials [96–98,102]. These figures are of the same magnitude as those of the most common allergens in the standard tray of contact allergens (Table 1). Thus, a new common cause of ACD was revealed.

Polyethers, e.g., ethoxylated surfactants and polyethylene glycols, are easily autoxidized [106–108]. Investigations of a chemically well-defined ethoxylated alcohol, penta-ethylene glycol mono *n*-dodecyl ether (Figure 12), showed that this type of compounds forms a complex mixture of autoxidation products when exposed to air. Predictive testing in guinea pigs revealed that the pure non-oxidized surfactant itself is not a sensitizer, but that the majority of investigated oxidation products are skin sensitizers [109–113] (Figure 12). Ethoxylated surfactants are non-ionic surfactants widely used in household and industrial cleaners, topical pharmaceuticals, cosmetics and laundry products. These surface-active agents are emulsifiers as well as suspending, wetting and solubilizing agents that give final products physical stability. Non-ionic surfactants are often preferred to ionic surfactants in topical products, since they are considered to cause less skin irritation, but autoxidation also increases the irritation [114]. Due to their irritating effect it is difficult to diagnose ACD to these compounds by patch testing [115]. However,

precautions must be taken in handling and storage of ethoxylated surfactants to avoid formation of allergenic mixtures.

5.2. Metabolism

The skin possesses the full armentarium to metabolize xenobiotica. Enzymes that are involved include cytochrome P450 (CYP) isoenzymes, flavin containing monooxygenases (FMO) as well as epoxide hydrases and transferases such as UDP-glucuronyltransferase or acetyl-*N*-transferase. In particular, in follicular keratinocytes more CYP-dependent enzyme activity was found compared to other keratinocytes that made it possible to demonstrate the inducibility of these enzymes by several xenobiotica in human skin under *in vivo* conditions [116]. The presence of multiple CYP enzymes in the skin was shown on mRNA, protein levels as well as on the catalytic level [117]. Reverse transcription-polymerase chain reaction (RT-PCR) revealed constitutive expression of cytochromes 1A1, 1B1, 2B6, 2E1 and 3A5 in keratinocytes and showed expression of cytochrome 3A4 after incubation with dexamethasone. The expression of cytochrome 1A1 was enhanced on the mRNA level after induction with benzanthracene [117]. mRNA data were confirmed on the protein level by immunoblots and immunohistology which showed expression of cytochrome P450 1A1, 2B6, 2E1 and 3A. Constitutive activity of CYP 1A1, 2B, 2E1 and 3A enzymes was measured by catalytic assays [117]. Also, flavin-containing monooxygenases were found in normal human epidermal keratinocytes (NEHK) — in particular the FMO3 [118,119]. Furthermore, transferases mediating the second phase of xenobiotic metabolism such as glutathione-*S*-transferases, *N*-acetyl-transferases or UDP-glucuronosyl transferases were detected in the skin [120–122].

Further studies showed that monooxygenases and transport-associated proteins such as multidrug resistance-associated transport proteins (MRPs) play complementary parts in drug disposition by biotransformation (phase I) and anti-transport (phase III) and act synergistically as a drug bioavailability barrier. RT-PCR analysis of membrane-associated transport proteins revealed constitutive expression of MRP 1 and MRP 3–6 in human epithelial keratinocytes, demonstrating that normal human keratinocytes express a cell-specific pattern of efflux transport proteins [117]. Using real-time PCR, RT-PCR, cDNA microarray, immunostaining and efflux assays it was shown that stimulation of normal human epidermal keratinocytes (NHEK) and primary human dermal fibroblasts with IL-6, in combination with its soluble α-receptor, or oncostatin M (OSM) resulted in an up-regulation of MRP expression and activity [123]. These results suggest a regulating control between the transformation of small molecular weight compounds and inflammatory skin disorders.

Expression of transport proteins involved in the uptake of xenobiotics has been detected on the mRNA and protein levels in human keratinocytes, revealing a different expression pattern as compared to primary liver cells [124]. The transporters studied included the subtypes A, B, C, D and E of the organic anion transporting polypeptide (OATP) family, which are responsible for the uptake of various anionic and neutral molecules and especially organic cations, including drugs.

Constitutive expression of OATP-B, OATP-D and OATP-E was shown in NHEK using RT-PCR and Northern-blot analysis, as well as in human skin tissue shown by tissue-blot hybridization and immunohistochemistry [124]. Expression of OATP-A and OATP-C was not detected in any of the keratinocyte samples. In contrast, liver tissue showed a significant expression of OATP-A and OATP-B as well as OATP-C, a weak expression of OATP-D, and no expression of OATP-E [124]. Using an uptake-transport assay, uptake of known and well-characterized OATP substrates like estradiol-17β-glucuronide and estrone sulfate was inhibited in NHEK by specific inhibitors such as taurocholate, verifying the functional capacity of the expressed OATPs. Even though the substrate specificity of the OATP isoforms is only partially known, these findings support the concept that uptake of large organic compounds like drugs or ACD antigens in keratinocytes is an active transport process mediated by members of the OATP family. The data confirm that NHEK express a specific profile of xenobiotica-metabolizing enzymes and transporters involved in drug influx and efflux. This may be important for the processing and presentation of small molecular weight compounds including drugs like carbamazepine, sulfonamides or contact allergens such as fragrances [125–127].

6. Chemical Aspects on the Most Common Allergens in the Standard Series

When looking at the allergens on top in the standard tray for screening of contact allergy from a chemical point of view, it is obvious that all are reactive compounds or can act as prohaptens (Table 1). Metal salts and especially nickel are the most common causes of contact allergy. This is not because the nickel ion is especially sensitizing but due to the massive exposure. Strict regulation of the exposure to nickel ions in items in close contact with the skin has shown that it is possible to reduce the frequency of nickel allergy in society [128]. Fragrance compounds are the most common allergens next to metal salts. Many fragrance compounds are activated by skin metabolism or by air oxidation but some are reactive enough to bind to proteins and are thus sensitizers themselves, e.g., cinnamic aldehyde that is one of the fragrance allergens included in the standard series. Also cinnamic alcohol is included. It is a prohapten of cinnamic aldehyde. However, in some cases contact allergy is diagnosed due to reactions seen only to patch testing with cinnamic alcohol. A challenge for future patch testing is to use oxidized fragrance terpenes for screening since the strongest allergens in the oxidation mixtures, the hydroperoxides, will decompose at normal handling in the dermatology clinics. The third most frequent group of sensitizers in the standard series is the preservatives. To act as a preservative or antimicrobial agent a compound needs to be reactive and thus it will also trigger our immune system. New potent preservatives e.g., MCI/MI (a mixture of 5-chloro-2-methyl-4-isotiazolin-3-one) and MI (2-methyl-4-isothiazolin-3-one) and MDBGN (1,2-dibromo-2,4-dicyanobutane) (Figure 6) were introduced in the market as very efficient antimicrobial agents. However, soon after their introduction in the market, frequent cases of ACD caused by these preservatives

were reported [77]. This lead to a strict regulation of their use in products in close contact with skin: MCI/MI is only allowed in a concentration of 15 ppm in such products and MDBGN is forbidden in all cosmetic products.

7. Predictive Testing

For pharmaceutical and cosmetic industries, it is mandatory to identify chemicals that are potential contact allergens before they become part of a new product and are introduced in the market. Today the murine LLNA represents the recommended and most widely used assay for the identification and potency assessment of contact allergens. However, alternative assays are being developed since there is an increasing demand from both legislative authorities and the society to reduce the need for experimental animals. A ban on *in vivo* testing of cosmetic and toiletry ingredients for skin sensitizing properties will come into force within the European Union in 2013 [129]. However, so far, no predictive *in vitro* methods robust and reliable enough to be used as stand alone screening methods for potential contact allergens are described in literature.

7.1. Animal models

Methods for determination of the skin sensitizing effect of a chemical have for many years used animal experiments. On the basis of the assumption that essentially all basic features of allergic contact reactions regarding macroscopic appearance, time course and histopathology are shared with humans, the mechanisms of contact allergy have been extensively studied in experiments in guinea pigs. Using various protocols, guinea pig experiments were designed to mimic ACD in humans and include both the induction and the elicitation phases [130]. The mouse ear swelling test (MEST) is also supposed to detect contact allergens by developing induction–elicitation phases [131]. Since mice are less sensitive than guinea pigs with regard to induction of contact allergy this method has not been useful in detection of moderate to weak allergens. A more recently developed predictive assay in mice, the LLNA [69], is based on the fact that a clear correlation exists between the vigor of a proliferative response induced in the local draining lymph nodes by topically applied chemicals and the extent of sensitization developed [132]. Results are expressed as mean dpm/lymph node for each experimental group and as stimulation index (SI), i.e., test group/control group ratio. Test materials that at one or more concentrations cause an SI of 3 or higher are considered to be positive in the LLNA. EC3 values (estimated concentration required to produce a SI of 3) are calculated by linear interpolation and used to compare sensitizing capacity of the different test materials [133]. The sensitizing potency of the test substances is classified according to the following: EC3 $<0.1\%$, extreme; EC3 $\geqslant 0.1\%$ to $<1\%$, strong; EC3 $\geqslant 1\%$ to $<10\%$, moderate; EC3 $\geqslant 10\%$ to $<100\%$, weak [134]. Thus, the end point in LLNA is the induction and

not elicitation of a local skin reaction as in ACD in humans or in the guinea pig tests. Extensive comparisons with guinea pig test data and with clinical experience have shown that the LLNA method is comparable to the guinea pig methods in the ability to predict correctly the sensitizing capacity of chemicals in humans [135]. The LLNA is now an acceptable alternative to predictive standard guinea pig methods, since it offers reliable hazard identification information. It is therefore used in risk assessment in industry [136]. As the method does not clearly discriminates between irritants and allergens [137], it is important to use the method with care and not classify irritant compounds as sensitizers based on a positive response in the LLNA at high concentrations. The major advantage of the LLNA is that it offers a possibility to determine a quantitative response of the sensitizing effect of a chemical, which makes the results from this method useful in QSAR studies.

7.2. *In vitro* testing

An *in vitro* approach to predict the skin sensitizing potential of a chemical should be designed to include the possibility to penetrate into viable epidermis, react with protein/peptides and initiate an antigen-specific immune response. Possible metabolism of inactive to active compounds must also be considered. Much research has been devoted to *in vitro* testing but with limited success [138]. Thus, there is still an urgent need for new method developments. A recent review regarding *in vitro* assessments of the sensitizing activity of low molecular weight compounds is given by Vandebriel et al. [139]. The possibility to use cultivated peripheral blood-derived DC was a significant improvement in strive to develop *in vitro* predictive assays [140,141]. Another step forward was the possibility to use real-time RT- PCR instead of direct measurements to investigate changes in the activation markers after induction with a contact allergen.

The novel *in vitro* assays are considered to allow assessments within human cell systems, which may be of advantage in particular with regard to the metabolism of prohaptens that is species dependent. Two cell types are predominantly involved in this process, i.e., LC, the professional antigen presenting DC in epidermis, and keratinocytes. Human DC have been employed to assess the sensitizing potential of various chemical agents *in vitro*, because exposure to contact allergens is known to alter both cell surface marker profiles and gene expression of immature DC [139,142]. Recently, Aeby et al. [143] demonstrated that DC exposure to the contact sensitizer 2,4,6-trinitrobenzenesulfonic acid induced a dose-dependent modulation of genes associated with DC maturation (i.e., IL-1β, AQP3), whereas exposure to the model irritant SDS did not entail significant gene regulation. Other studies corroborate the idea of IL-1β and AQP3 as specific markers of contact allergen-associated DC activation and propose further candidate genes such as CCR6, CXCR4 [142] and CCL2 [144]. Of note, marked IL-8 up-regulation has been shown to occur during DC exposure to potent contact allergens [145,146].

Several studies have used keratinocytes to evaluate the sensitizing capacity of low molecular weight chemicals, showing that intracellular IL-1α may be a first parameter to identify sensitizers *in vitro* using keratinocytes [139]. Also, the

distinction between sensitizers and irritants has been a subject of several investigations mostly utilizing IL-1α, but also IL-8, IL-12, and TNF-α expression as indicators of xenobiotic-induced cell activation [139]. Studies by Lisby et al. [147] revealed that some irritants and irritant sensitizers directly induce TNF-α in keratinocytes without intermediate LC-derived signals. However, additional parameters may be required to evaluate fully this approach.

So far, only potent sensitizers have been identified by *in vitro* methods. Since most contact allergens have a weak or moderate sensitizing effect this is a challenge for future work. The water solubility of the chemical to be investigated is a trivial but fundamental problem in all cellular-based assays, since most of the skin sensitizers identified are organic compounds with low water solubility. Another problem is the stability of the compound in the cellular experiments that are performed over many hours and days. It is therefore not surprising that a majority of results presented in literature from *in vitro* experiments describe results from experiments with Ni ions. However, it is not possible to draw general conclusions on the mechanisms of contact allergy based only on Ni^{2+} experiments where a specific antigen presentation seems to take place [12–14].

Detailed characterization of CYP expression and functional activity in human skin and skin cells is a prerequisite for understanding the role of these enzymes in drug metabolism and carcinogenesis. In addition**,** recent studies indicate that some CYPs may play an important role in the metabolic activation of prohaptens into peptide bound antigens that can be presented to LC [148]. On the basis of the microarray and quantitative reverse transcriptase-PCR studies of the CYP content in human skin samples, an enriched skin-like recombinant human (rh) CYP cocktail using CYP1A1, 1B1, 2B6, 2E1 and 3A5 was established [149]. To validate this rhCYP cocktail, metabolism of a recently described [150] model prohapten ((5*R*)-5-isopropenyl-2-methyl-1-methylene-2-cyclohexene) was investigated. This prohapten was converted to reactive epoxides in all non-cell-based incubations including the skin-like rhCYP cocktail. This rhCYP cocktail therefore provides a simplified alternative to using skin tissue preparations in mechanistic studies of CYP-mediated skin metabolism of prohaptens. It may also offer future possibilities for designing *in vitro* predictive assays for assessment of allergenic activity of prohaptens, e.g., in combination with keratinocyte- or DC-assays. With the discovery of new antibodies as well as novel CYP enzymes, the skin-like rhCYP cocktail may in the future continually be improved to provide an even better model for physiological conditions of human skin.

7.3. *In silico* methods

Many experiments show a clear correlation between the inherent reactivity of a compound towards nucleophilic groups in skin proteins and its sensitizing capacity. The protein reactivity of compounds is an important property in the development of QSAR models for skin sensitization. In addition also other properties of a chemical (e.g., molecular volume, log *P*, pK_a) can be analyzed and incorporated into (Q)SARs. Rule-based expert systems where chemical structures are related to a specific outcome are also available. Deductive Estimation of Risk from Existing Knowledge (DEREK for Windows) is one example of such a knowledge-based

expert system [151]. It covers a variety of toxicological outcomes and is well known within the field of skin sensitization research. New compounds can be analyzed for structural alerts by comparing with existing data in the expert system, i.e., the presence of substructures associated with a certain type of toxic activity. The system is continuously updated but is never better than the current knowledge [152]. Such expert systems are useful in the initial screening of the potential sensitization hazard of a chemical but cannot be used as stand alone risk assessment tools in safety support. For compounds that need to be activated either by air oxidation or by skin metabolism, the relationship between chemical reactivity and sensitizing capacity is less straight forward and the incorporation of these features is a challenge in the improvement of *in silico* methods. Also, radical reactions and complex formations besides the nucleophilic–electrophilic interactions between haptens and proteins must be considered in future *in silico* methods.

8. Outlook

An acquired contact allergy is life long and still only symptomatic treatment of ACD exists. Thus, prevention from exposure to skin sensitizing chemicals is of utmost importance. The ultimate challenge for developing non-animal test methods is applying our mechanistic understanding of contact allergy and ACD to the design of predictive *in vitro* alternative test methods. Since environmental exposure is of major importance for the development of ACD, regulatory work and legislation to decrease or forbid exposure of potent contact allergens are important. A review with historical examples on actions to control contact allergy epidemics was recently published [153]. For nickel and chromium (as chromate), strict regulation of exposure has caused a decrease in new cases of ACD [153]. Also, regarding fragrance allergy there is an ongoing regulatory work to try to diminish the exposure of allergenic fragrance compounds. However, regulations of exposure to these compounds are much more difficult. Numerous compounds with different chemical structures are used as fragrances, they are found in natural products in complex mixes, and some compounds easily autoxidize and form highly potent contact allergens. The increased awareness and the regulatory work of the most important fragrance allergens have decreased their usage in perfume compositions [154] and also a decrease in the number of positive test reactions to the fragrance mix used for screening in consecutive dermatitis patients is reported [155]. An important issue is that so far, all data are based on single compounds but what happens in the mixed products we are using as consumers? Increased knowledge of the mechanisms of contact allergy and improved methods to investigate them are therefore an ultimate need both in industrial production and regulatory work.

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Footnote to Ref. 155:
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CHAPTER 5

Inorganic Molecular Toxicology and Chelation Therapy of Heavy Metals and Metalloids

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Contents

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1. Introduction

The toxic properties of heavy metals and metalloids have been known for centuries. For example, the toxic nature of arsenic compounds was understood by the ancient Greeks and Romans, and they have been notorious throughout history as a homicidal and suicidal agent. Mercury compounds also have a long history of use as poisons. The medieval alchemist and physician Paracelsus (1493–1541) has been called the father of modern toxicology, and one of his most famous quotes is translated as "only dose makes the poison". It is now well understood that Paracelsus was in part correct. However for heavy metals and metalloids it is not *only* the dose, but also the molecular forms in which the metal or metalloid is present that are critical in conferring toxic properties. Thus, both arsenic and mercury compounds have widely varying toxicities, and indeed both elements have also seen use in medicine (Paracelsus was in particular an advocate of the supposed benefits of mercury) as well as poisons. While many of the medicinal uses of these metals have been discredited, their clinical use was only possible for some of the less toxic chemical forms. This review will discuss the bioinorganic chemistry underlying the molecular toxicology of heavy metals and metalloids, with particular reference to mercury and arsenic. It is not intended to be a comprehensive review of the entire field of bioinorganic molecular toxicology, but instead a focused examination of selected areas to illustrate the application of the *in situ* spectroscopic technique of X-ray absorption spectroscopy (XAS), and the use of quantum mechanical molecular modeling in toxicology.

2. *In Situ* Probes of Molecular Form

2.1. X-ray absorption spectroscopy — an *in situ* probe of molecular structure

XAS is a powerful tool for investigation of both physical and electronic structure [1]. As spectroscopic methods go it is a relatively new technique, originating in the early 1970s, and in recent years it has become increasingly applied to a very wide variety of fields. The major strength of XAS is that it can be used to obtain molecular information on a sample with essentially no pre-treatment. Moreover, the sensitivity has increased dramatically in recent years, and a few groups now routinely investigate samples with micromolar levels of metals or metalloids [2], whereas previously millimolar levels were required. This new sensitivity allows investigation of physiologically relevant levels of metals and metalloids *in situ* within tissues for the first time.

X-ray absorption spectra arise from core-level excitation by absorption of X-rays, and are thus associated with an absorption edge (e.g., 1 s excitation for a K-edge, Figure 1). They are usually separated into two different regions: the Extended X-ray Absorption Fine Structure or EXAFS, which occurs at energies higher than the absorption edge; and the near-edge region which consists of features before the major inflection, and any after the inflection which are not a

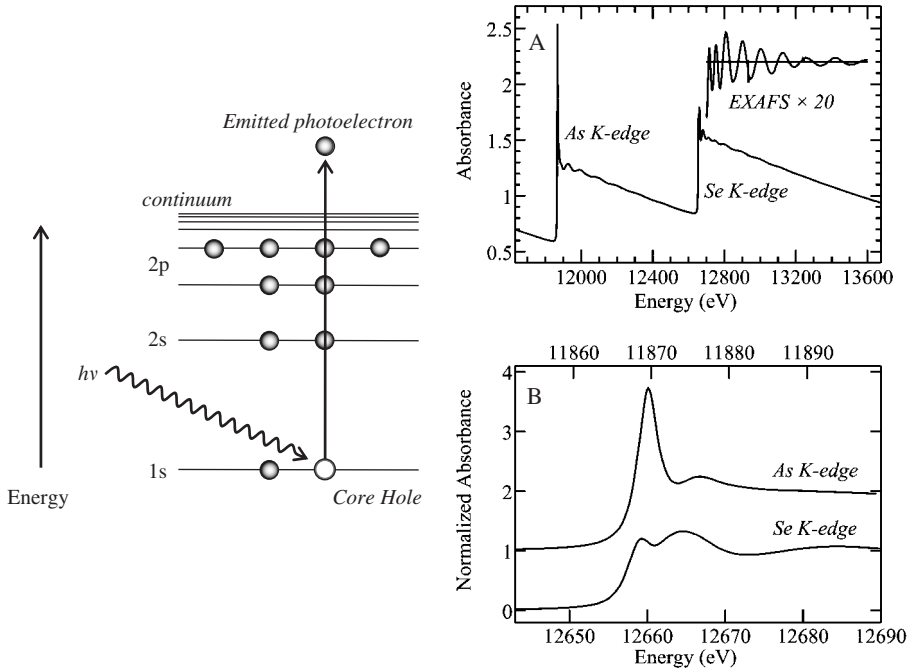


Figure 1 (left) Schematic diagram of X-ray photo-absorption event in which an X-ray photon (*hv*) is absorbed by excitation of a 1 s electron to create a core hole. (right) A shows the raw data from the synthetic seleno-bis(*S*-glutathionyl)arsinium ion, with the As and Se K-edges indicated, and the extracted EXAFS oscillations shown in the inset. B shows the As and Se near-edge spectra, which are simply the K-edge absorption region from A, background subtracted, normalized and re-plotted with a narrower energy range.

part of the analyzable EXAFS. XAS is element specific and can be used to investigate solids, liquids (including solutions), gaseous materials, and any mixtures thereof. XAS probes all of an element within a sample with moderate sensitivity, and is applicable to a very wide range of elements. XAS theory is well developed, as are the methods of analysis of the EXAFS part of the spectrum to give a local radial structure for the absorbing atom. In essence, the EXAFS can be used to approximately determine what the neighbor atoms of the absorber atom are (i.e., their approximate atomic numbers), how many there are of a given type, and very accurately estimate inter-atomic distances. Figure 1 shows a schematic of the physics of XAS showing both the EXAFS and near-edge regions. A detailed discussion of XAS analysis methods is outside the scope of this review, and has been presented elsewhere [1].

The sensitivity of the near-edge spectrum to electronic structure means that it can be used as a fingerprint of a particular chemical type. The sensitivity of the As K near-edge spectrum to arsenic chemical form is illustrated in Figure 2. Each chemical species presents a subtly different spectrum and this can be used to obtain information on species present *in situ*. In many cases mixtures of species will be

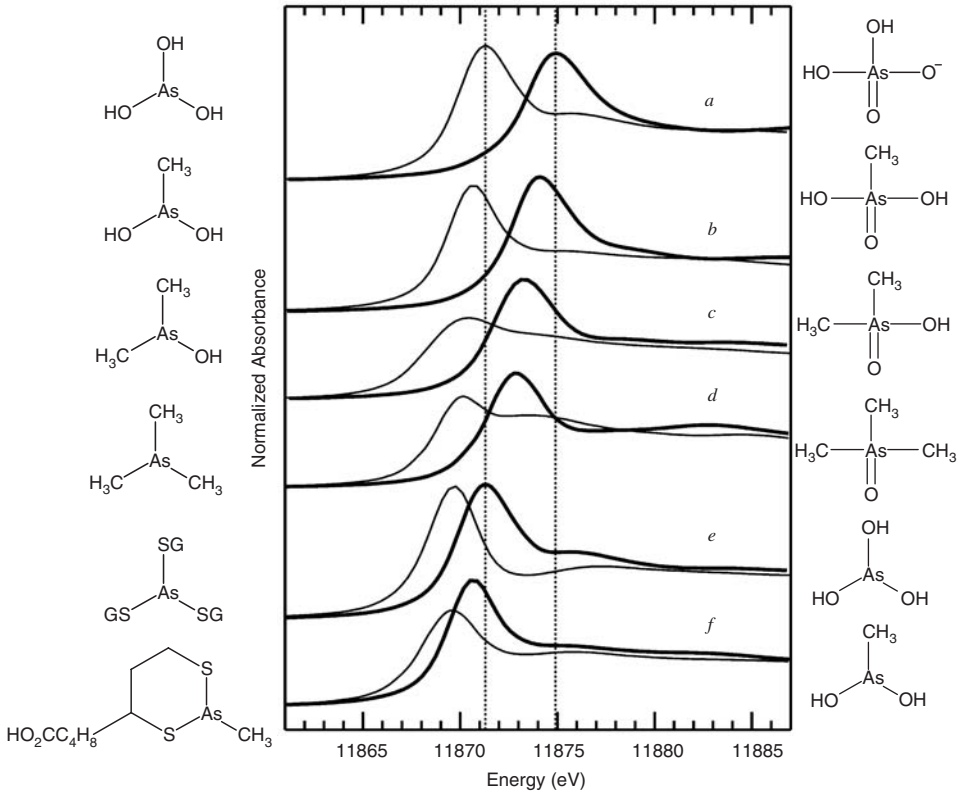


Figure 2 Sensitivity of X-ray absorption near-edge spectra to chemical form illustrated using different As^{III} and As^{V} compounds. Spectra are plotted as pairs of related species, *a*–*d* compare As^{III} and As^{V} species as narrow and bold lines, respectively, while *e* and *f* compare different As^{III} compounds. In *e* arsenic tris-*S*-glutathione (narrow line) is compared with arsenite (bold line), and in *f* the lipoic acid (1,2-dithione-3-pentanoic acid) (narrow line) complex with methylarsonous acid is compared with methylarsonous acid (bold line). The vertical lines are drawn to guide the eye to shifts in the spectra.

present, and techniques have been described to quantitatively analyze mixtures using least squares fitting of a linear combination of model spectra, or principal component analysis [1]. It is important to note that the technique does not provide an exact identification, but rather a probe of the environment of the metal or metalloid. For example, selenomethionine and Se-methyl-selenocysteine both have similar local selenium environments (specifically CH_3–Se–CH_2–), and as a consequence give essentially identical near-edge spectra [3]. These species cannot be distinguished by XAS, and other techniques must be used to provide this information. Another restriction in application of this type of analysis is that spectra of a model species with a similar coordination to the unknown must be at hand, and in the case of truly new or novel species these may not be available. In this case EXAFS spectra can be used to provide details of the coordination

environment, which requires more lengthy data acquisition strategies. Despite these limitations, an *in situ* probe of chemical form can be a powerful tool for research in molecular toxicology, and we predict that applications will increase as the sensitivity of the technique continues to improve.

2.2. X-ray microprobe and XAS imaging

Biological samples typically have detailed anatomical or spatial structure, and the elemental and chemical distribution of metals and metalloids with respect to that structure is of significant interest. X-ray microprobe and XAS imaging techniques can be used to provide such information. Here a micro-focused X-ray beam is used to illuminate the sample, which is raster scanned while the X-ray fluorescence is monitored, so that an image or map of the sample is built up. Methods of quantitative analysis have been described [4,5] in which quantitative concentration maps of particular chemical species can be generated. To date XAS imaging has been little applied in toxicology, with the notable exceptions of studies designed to track chromium biotransformations on a sub-cellular level [6].

2.3. Computational chemistry

The availability of fast, highly accurate quantum chemical codes has revolutionized some approaches to structural chemistry. Indeed, density functional theory (DFT) is now among the most powerful tools available to the computational chemist, and the recent availability of these efficient, rigorous, and powerful codes has revolutionized the field of quantum chemistry (Walter Kohn and John Pople shared the 1998 Nobel Prize in Chemistry for the development of DFT). Unlike older codes, which cannot reliably handle heavy atoms such as mercury, DFT can be used to compute the three-dimensional structures of molecules involving any atom. The upsurge in use of DFT is evident from the number of citations in Chemical Abstracts, which increased from several hundred per year in the early 1990s, through more than 1,000 in 1995, to more than 10,000 in 2006. DFT can calculate structures starting from postulated arrangements of atoms to arrive at accurate structures that can be expected to be accurate. These calculations typically adjust the structure to minimize the computed energy of a molecule using an iterative procedure. The computation time per energy calculation increases as approximately the square of the number of electrons in the structure, and for large molecules (i.e., with more than 100 atoms) one either needs a very powerful computer (and some patience) or to employ more approximate methods. With even larger molecules the time required for computation becomes prohibitively large, and in these cases currently one must resort to more approximate methods such as semi-empirical codes or molecular mechanics. Figure 3 illustrates the accuracy of DFT calculations by comparing the results of an energy minimization calculation with the structure determined from X-ray crystallography. The computed and crystallographic structures are essentially identical, and the small differences that do exist can be attributed to crystal packing forces in the crystalline sample. DFT can be used to answer fundamental questions about bonding. It also provides the possibility of a

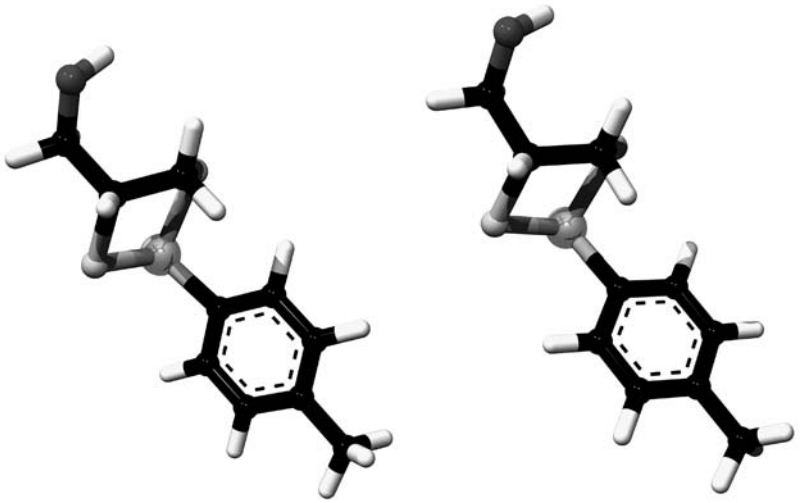


Figure 3 Spot the difference — crystallography (left) vs. density functional theory (right), showing near identity of the structures. Any minor observed differences can be attributed to such effects as crystal packing forces.

computational reality check, in that it can easily test whether a proposed structure is chemically reasonable or not.

3. Chemical Form and Function in Toxicology

Knowledge of the molecular form in which a toxic metal or metalloid is presented is essential for understanding toxic response. Indeed, every aspect of how a toxic metal or metalloid is transported within the environment and taken up into living things and whether or not it is toxic, benign or even beneficial is determined by the molecular form. This is well illustrated by the arsenic content of fish. Marine fish contain approximately 0.02% by weight of arsenic, but present little or no toxic hazard, because the compounds present (the As^{III} zwitterions arsenobetaine and some arsenocholine) are not metabolized. Conversely, other As^{III} species are known to present a variety of toxic responses. When it comes to understanding toxicological molecular mechanisms, knowledge of the form in solution, or even in the tissue itself, is very important.

3.1. The flexible coordination chemistry of mercury

Mercury exists in metallic Hg^0 or mercuric Hg^{II} oxidation states and both are important biologically. Mercurous species, containing the Hg_2^{2+} cation, with a strong metal–metal bond, are not known to be important in biological systems. This discussion is restricted to the coordination chemistry of mercuric species.

The coordination chemistry of mercuric mercury has been called fictile [7] — the metal can exist in a number of alternative coordination environments with little thermodynamic preference between them. For example, mercuric ions form three discrete solution species with chloride: the linear $HgCl_2$ molecule, which in solution with excess Cl^- forms the anionic species $[HgCl_3]^-$ and $[HgCl_4]^{2-}$. These have preferred geometries typical of mercury: linear diagonal, trigonal planar, and tetrahedral, respectively (Figure 4). In sea water, which typically has a pH of around 8.3 and a chloride concentration of 0.55 M, mercuric ions would be predominantly present as the tetrahedral $[HgCl_4]^{2-}$. In other environmental systems various mercuric species will exist, including $HgCl_2$, HgCl(OH), $Hg(OH)_2$, and $[Hg(OH)_3]^-$. In fresh drinking water which should typically have less than 4 mM Cl^-, and a pH ranging from 6 to 8, mercury will be present as a mixture of $HgCl_2$, HgCl(OH), and $Hg(OH)_2$. The affinity of Hg^{2+} species for thiolates is well-known, indeed the old name for thiolate, mercaptan, derives from *mercurius captans* or "laying hold of mercury", and much of the biological activity of mercury is thought to be associated with thiolate complexes [8]. These characteristics are shared by the biologically important cuprous ion Cu^+, whose valence shells are isoelectronic with Hg^{2+}. For example, linear two-coordinate and trigonal planar cuprous thiolate species are commonly found in

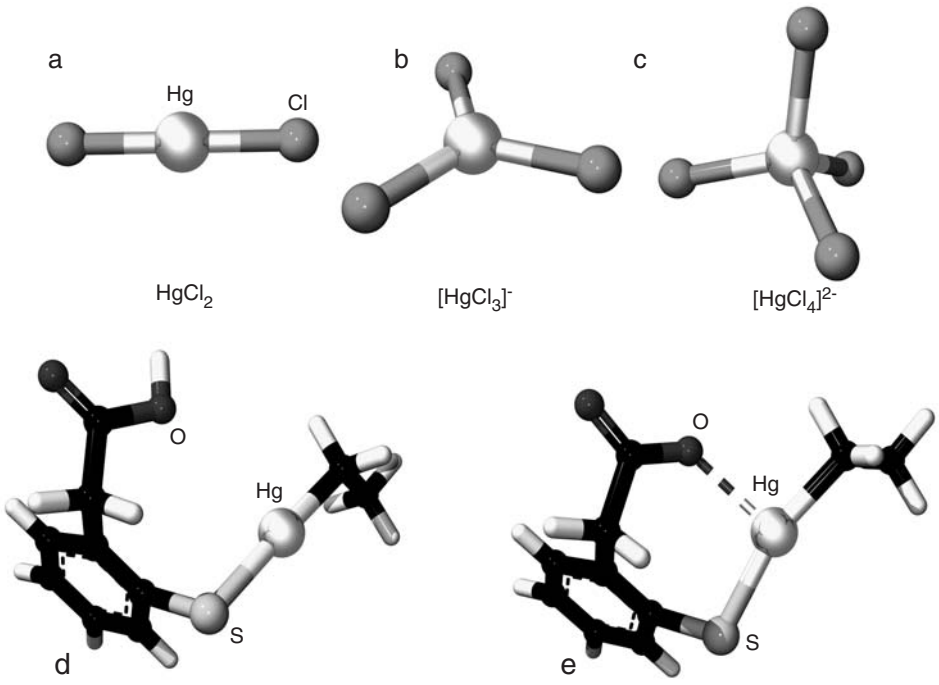


Figure 4 Coordination geometry of mercury compounds: a shows the structure of the neutral $HgCl_2$ molecule and b and c the structures of the $[HgCl_3]^-$ and $[HgCl_4]^{2-}$ anions, respectively. d and e show computed structures for thimerosal in protonated and deprotonated forms, respectively.

copper metallochaparones [9], transcription factors [10,11] and copper transporters [12,13]. We will return to this point in the section on custom chelators, below.

Less well-known than mercury's affinity for thiols, but perhaps also important, is the high affinity mercury has for transition metal ions; mercury forms strong metal–metal bonds with a variety of transition metal ions, and this chemistry is well investigated [14], the toxicological implications of this chemistry remain essentially un-explored. In addition to the simple coordination environments described above, mercury also often adopts a T-shaped coordination, with two short bonds and a third longer bond. This should really be considered a special case of the linear coordination. One example of such coordination is to be found in the controversial drug additive thimerosal [15], which can be shown using DFT to adopt a linear two-coordinate geometry when the carboxylic acid is protonated, but a three-coordinate T-shaped coordination in the deprotonated form (Figure 4).

Much applied toxicology of mercury focuses on methylated derivatives. These are important both because they are neurotoxic, and because there is widespread exposure of human populations through dietary intake. In principal all the mercury coordination environments described above can occur with methylated derivatives, however, to date, no *bona fide* three-coordinate trigonal planar or four-coordinate tetrahedral species have been described. Species containing mercury coordinated to one methyl group are commonly referred to as methylmercury species. There is a slightly confusing nomenclature that is common in the toxicology literature, as according to standard chemical nomenclature "methylmercury" is actually what is commonly called "dimethylmercury" (CH_3–Hg–CH_3). Here we adhere to the common usage in which methylmercury contains only a single methyl, with one or more other ligands to the mercury (i.e., CH_3Hg–R). It is also common practice to denote aqueous solutions of methylmercury compounds, for example methylmercury chloride, as containing CH_3Hg^+, but this cation probably never actually exists in solution. In the case of chloride (for example) the Cl^- will either remain strongly bound to Hg in solution, or alternatively exchange for a different ligand such as OH^-.

3.2. Arsenic coordination chemistry

Arsenic is found in As^{III} and As^V oxidation states in biological systems. The coordination geometry of As^V is mostly pseudo tetrahedral. While uncommon, six-coordinate arsenates with octahedral coordination geometry do exist [16], and a number of other six-coordinate As^V complexes with approximately octahedral geometry have been described, some with diol ligands to the arsenic [17].

Trivalent arsenic is usually three-coordinate with pyramidal geometry, whereas pentavalent arsenic is four-coordinate with approximate tetrahedral geometry. We note in passing that although five- and six-coordinate As^V species are quite common in the chemical literature, these are typically not found in biological systems. As^{III} is of special interest because of its toxicological activity, and we will consider its compounds. The oxy-acid arsenite is often written as $[AsO_2]^-$ although no such species actually exists. This misunderstanding arises from the formula for sodium arsenite, which is usually written as $NaAsO_2$, whereas in fact

the crystal structure consists of polymeric chains of $As(O)_3$ pyramids, linked by oxygens, with Na^+ cations positioned between the chains. Arsenite is always a pyramidal species and in aqueous solution at physiological pH is present essentially as $As(OH)_3$. Like Hg^{II}, As^{III} has a high affinity for thiols, and a somewhat higher affinity for selenium, and this characterizes much of its biological chemistry. Examination of the Cambridge Structure Database indicates that relatively few As^{III} thiolato compounds have been structurally characterized, and no complexes involving biological ligands such as cysteine or glutathione have been reported. As^{III} typically has a characteristic As–S bond-length of 2.27 Å, with an S–As–S bond-angle of 96°. The chelation agent diethyldithiocarbamate forms an apparently six-coordinate species with As^{III}, but three of the six As–S bonds are of normal length (~2.35 Å) and form pyramidal geometry, whereas the other three are much longer (~2.8 Å). These complexes can thus be considered as special cases of three-coordinate species. There are a few genuine instances of *bona fide* six-coordinate As^{III}, but these are not formed with biologically compatible ligands. Trivalent arsenic can also form tetrahedral arsonium species $[R_4As]^+$. These are present in the biomolecules arsenocholine and arsenobetaine that are found in abundance in seafood, and are not toxic to mammals.

4. Molecular Mimicry — A Case of Mistaken Identity

Molecular mimicry occurs when one molecular entity is "mistaken" for another by cellular or other biological processes. This concept is important in the development of autoimmune responses with some infectious diseases. In these cases, antigenic determinants of the infectious microorganisms resemble structures in the tissue of the host, but differ enough to be recognized as foreign by the host immune system [18]. A quite different molecular mimicry, of certain carbohydrates by specific peptides, has been suggested for possible drug use [19]. Molecular mimicry is also thought to be an important mechanism by which toxic metal species are taken up into living tissues [20,21], and arises from the toxic species being mistaken for one normally present. The root of this molecular mimicry is structural similarities between the two species in question. A simple example is provided by univalent thallium species. Th^+ has approximately the same ionic radius as K^+ (1.64 Å vs. 1.52 Å, respectively) and prefers weak ionic coordination. It is thus toxic because it is "mistaken" for K^+ in biological processes [22], being actively taken up and interfering with a variety of systems, including the nervous system [23]. Thallium is removed to the gut by excretion through the intestinal wall [24] but is subsequently actively taken up, primarily in the colon. This cycle of excretion and re-absorption can be broken by introduction of the polymeric substance known as Prussian blue (potassium ferric ferrocyanide) in which $[C{\equiv}N]^-$ groups bridge a cubic lattice of Fe^{2+} and Fe^{3+} octahedra (Fe^{2+}–$C{\equiv}N$–Fe^{3+}). The large vacancies in this structure are filled by water and K^+ ions. On introduction into the gut the vacancies in Prussian blue are preferentially filled by Tl^+, effectively breaking the cycle of absorption and re-excretion.

4.1. Oxyanions — genuine molecular mimicry

The most valid examples of molecular mimicry in inorganic chemical toxicology are the oxy-anions. As pointed out by Wetterhahn-Jennette [20], many tetrahedral oxy-anions closely resemble one another, and are thus hard to distinguish biochemically. For example, arsenate enters yeast cells using the phosphate-transport system [25], and arsenate mimics phosphate in the human red cell sodium pump and anion exchanger [26]. Both arsenate and phosphate are approximately tetrahedral oxy-anions, and are a mixture of mono and di-anionic forms at physiological pH ($[H_2PO_4]^-$ and $[HPO_4]^{2-}$ vs. $[H_2AsO_4]^-$, $[HAsO_4]^{2-}$). The corresponding phosphorus and arsenic anions have very similar dimensions, with arsenate being about 10% larger than phosphate (As–O bonds are $\sim$0.15 Å longer than corresponding P–O bonds) and thus arsenate is effectively a molecular mimic of phosphate. Similarly, chromate $[CrO_4]^{2-}$ and sulfate $[SO_4]^{2-}$ are very similar tetrahedral anionic molecules with chromate being about 12% larger than sulfate, and likewise chromate has been shown to be taken up by the sulfate transport system [27,28]. These tetrahedral oxy-anions can thus be regarded as genuine molecular mimics. However we note that molybdate $[MoO_4]^{2-}$ is some 19% larger than sulfate, and this difference is sufficient for the molybdate transporter which uses a rigid anion-binding pocket to discriminate between molybdate and sulfate, preferentially binding the former [29]. Loose-fitting sulfate is discriminated against mainly because of a greater desolvation penalty [29], and because the large size of the cavity prevents stabilizing interations. Sulfate transporters can effectively exclude molybdate because their anion-binding pockets are too small [29]. We note that molecular mimicry may occur even when there is little obvious molecular similarity. An example of this is given by the sodium iodide symporter, whose function is to transport iodide into the cell, and it does this by co-transporting two Na^+ ions along with a single I^-, with the transmembrane sodium gradient serving as the driving force for anion uptake [30]. The sodium iodide symporter will also take up anions such as perchlorate $[ClO_4]^-$, pertechnetate $[TcO_4]^-$, and perrhenate $[ReO_4]^-$ [31], and this has been exploited in radiotherapy of thyroid cancers using $[^{99m}TcO_4]^-$ [31]. At first glance the idea that this might be molecular mimicry makes little sense, but I^- is a large anion, with an ionic radius of 2.06 Å, and the monovalent oxy-anions discussed above have short bonds to oxygen (Cl–O, Tc–O, and Re–O are 1.405, 1.685, and 1.703 Å, respectively) with their single negative charges delocalized over the anion. Examination of the molecular surfaces indicates a degree of similarity between these species (Figure 5 compares the computed molecular surfaces for iodide and for perchlorate) which presumably, in the case of the sodium iodide symporter, is sufficient to allow uptake of the oxyanions *via* molecular mimicry.

4.2. Mercury transport — mimicry or not?

One well-accepted example of molecular mimicry is the transport of methylmercury-cysteine [32], which is thought to mimic the amino acid methionine, thereby gaining entry into the cell *via* an amino acid carrier, the LAT1 transporter [33].

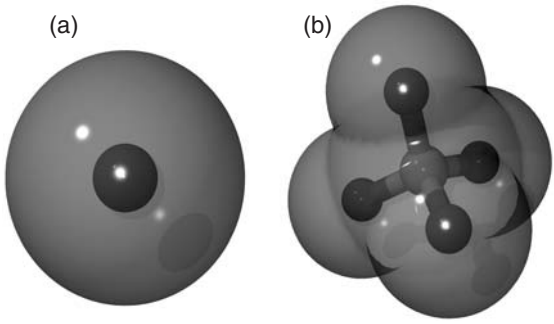


Figure 5 Comparison of molecular surfaces (shown as translucent surfaces) for iodide (a) and perchlorate (b), shown at the same scale.

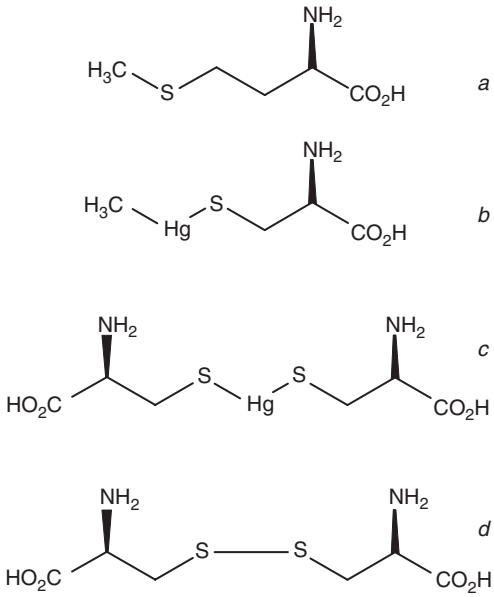
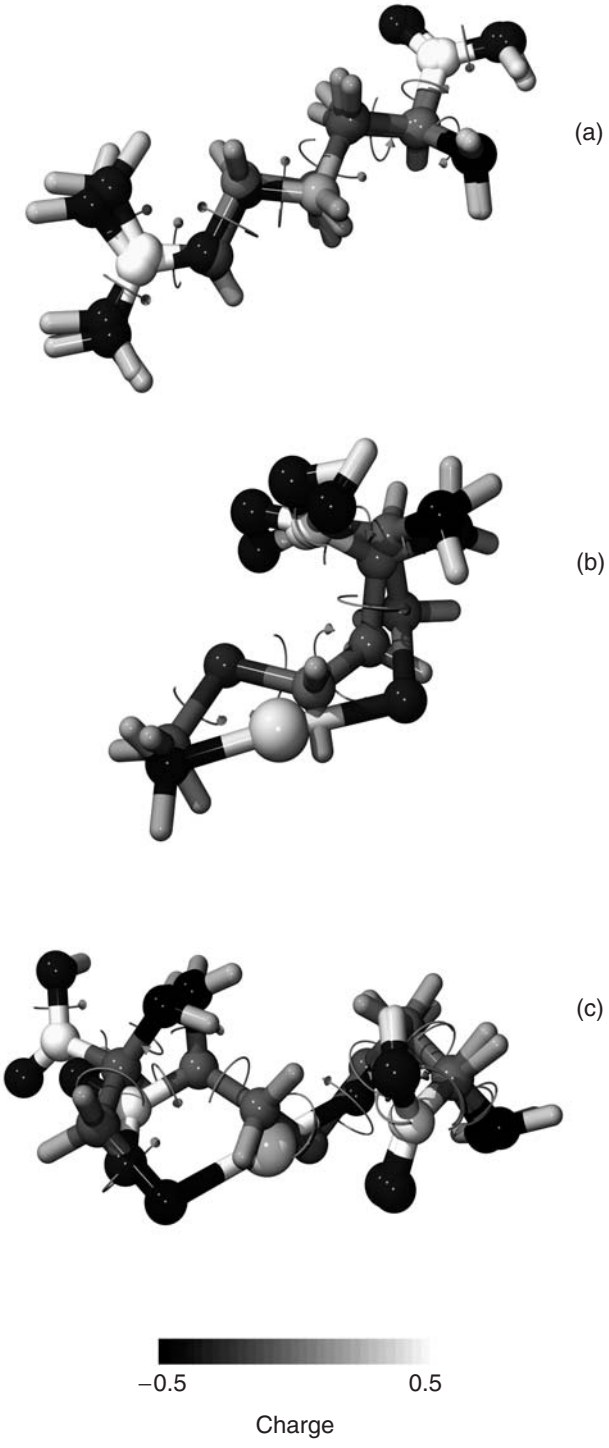


Figure 6 Schematic structures for *a* methionine, *b* L-cysteinato(methyl)mercury(II), *c* mercury(II) bis-L-cysteineate, and *d* cystine, showing structural similarities that have been posited between mercury compounds and natural products.

The structural similarity that has been supposed to exist between methionine and methylmercury-cysteine is demonstrated in Figure 6, which has been deliberately drawn with an erroneous metal geometry so as to illustrate the arguments of structural similarity underlying the proposed molecular mimicry. It has also been assumed that molecular mimicry plays an important role in the toxicology of a number of different mercury species [21].


(a)
(b)
(c)
−0.5
0.5
Charge

As we have discussed, molecular mimicry involves possible similarities between molecules. Quantitative molecular comparisons can be made using the method of Hodgkin and Richards [34], which can take into account both molecular shape and charges on component atoms. The Hodgkin index H_{AB} is given by Equation (1), where ρ is the property being considered, the integration is over space, and A and B refer to the two different molecules being compared.

$$H_{AB} = \frac{2\int \rho_A \rho_B \mathrm{d}v}{\int \rho_A^2 \mathrm{d}v + \int \rho_B^2 \mathrm{d}v} \quad (1)$$

In order to take into account both steric and electrostatic fields, the similarity index H can be evaluated as an equally weighted sum of Hodgkin indices for the steric and electrostatic fields, with the latter obtained from Mulliken atomic partial charges computed by DFT. Values for the similarity index H can thus range from -1.0 to 1.0, with 1.0 indicating a perfect overlap between fields, zero indicating no overlap, and -1.0 only occurring when electrostatic potentials and molecules are inversely aligned. Molecular similarities can be judged using this method employing energy-minimized structures from DFT as starting coordinates, allowing for conformational flexibility expected in solution arising from bonds that can rotate (i.e., searching among allowed conformations), and refining coordinates to obtain the highest possible H, which corresponds to the most perfect molecular match. For most systems it is not possible to get a condition corresponding to these conceptual limits of perfectly inversely aligned charge distribution or no overlap (where $H \rightarrow -1.0$ or 0.0), so that values of H always tend to be close to +1.0. Thus, the arsenate anion $[HAsO_4]^{2-}$, which is thought to be a molecular mimic for phosphate $[HPO_4]^{2-}$, gives a similarity index H of 0.97, while chromate $[CrO_4]^{2-}$ and sulfate $[SO_4]^{2-}$ give H of 0.99. The example of canavanine, a toxic amino acid present in Jack Bean, which is a molecular mimic of arginine [35] is shown in Figure 7a. This molecular mimicry is the basis for canavanine's biological activity, and L-canavanine and L-arginine give a similarity index of 0.99 (Figure 7a). To further calibrate the technique obviously dissimilar amino acids can be compared which tend to give values of H close to 0.8, for example L-tryptophan and L-methionine give a $H = 0.79$, whereas L-histidine and L-methionine give $H = 0.93$.

These methods have recently been used to objectively investigate molecular mimicry for two different mercury species, L-cysteinato(methyl)mercury(II) (called methylmercury cysteine) and mercury(II) bis-L-cysteinate [36,37]. Methylmercury cysteine was compared with the proposed target of its molecular mimicry

Figure 7 Calculated molecular superpositions showing the closest molecular match for (a) L-arginine and L-canavanine, (b) methionine and L-cysteinato (methyl)mercury(II) of methionine to L-cysteinato (methyl)mercury(II), and (c) cystine and mercury(II) bis-L-cysteineate. The atoms are colored according to Mulliken partial atomic charge computed from density functional theory. The arrows indicate the rotational degrees of freedom used in matching the two structures. Clear miss-match between the mercury species and the species that they are supposed to be mimicking is evident from both structural and electrostatic perspectives.

methionine, and the other LAT1 amino acids, and mercury bis-cysteinate with cystine (Figure 7b, c). Both show very poor matches with the amino acids that they have been proposed to mimic ($H = 0.85$ and 0.86, respectively). This work clearly demonstrated that there is no *a priori* chemical basis for the molecular mimicries that have been suggested for these compounds. It is not disputed that they are transported by amino acid transporters, but the lack of molecular similarity means that specific molecular mimicry is not the underlying mechanism (e.g., methylmercury cysteine is not a probable molecular mimic of methionine). We note that while this provides a quantitative and objective approach, it could clearly be improved, perhaps by constrained DFT refinements of each conformation being tested.

5. Synergism and Antagonism

5.1. Arsenic and selenium: Two wrongs that can make a right

The interaction of selenium with arsenic and with mercury (see below) has recently been reviewed in depth by Gailer [38], and we will therefore restrict our discussion of this here. The two most toxic environmentally common oxy-anions of arsenic and selenium are arsenite and selenite, respectively. When administered in isolation these have approximately equal toxicities, but when given together their toxic effects cancel. This antagonism was first reported nearly three quarters of a century ago [39]. The molecular basis of this surprising phenomenon was uncovered by XAS [40]; a novel arsenic-selenium compound — the seleno-bis (*S*-glutathionyl)arsinium ion, or $[(GS)_2AsSe]^-$ (Figure 8) is formed in erythrocytes [41], and subsequently excreted in the bile [42], presumably by action of a glutathione-linked transporter system [43][44]. The core of the species contains a terminal selenide, and possesses a single negative charge (note that the abbreviation $[(GS)_2AsSe]^-$ ignores possible charges on the glutathione ligands). The bond-length between As and Se is 2.32 Å, indicating a bond order of approximately 1.5 [40]. The formation of $[(GS)_2AsSe]^-$ is currently thought to be chemical, according to the scheme shown in Figure 9, and not mediated by enzyme catalysis [38], although we note that there is at present no proof of this.

While there are proponents of a biological role for arsenic [45,46], this is not proven, and the weight of modern opinion seems to be that arsenic has no defined biological roles. Selenium, however, is an essential micronutrient for mammals and birds, with known functions in a variety of systems. It is required for both intra and extracellular glutathione peroxidases, iodothyronine deiodinase, and thioredoxin reductase, as well as a number of selenoproteins with less well-defined physiological roles (e.g., selenoproteins P and W) [47]. In all these cases the selenium is present as the amino acid selenocysteine, which, where its role is understood, is involved in the catalytic active site of the enzyme. Selenium is also said to provide protection against various cancers including prostate cancer [48], colorectal cancer [49], lung cancer [50], and various gastrointestinal cancers [51]. Lack of dietary selenium has also been shown to increase susceptibility to infectious disease, such as influenza [52].

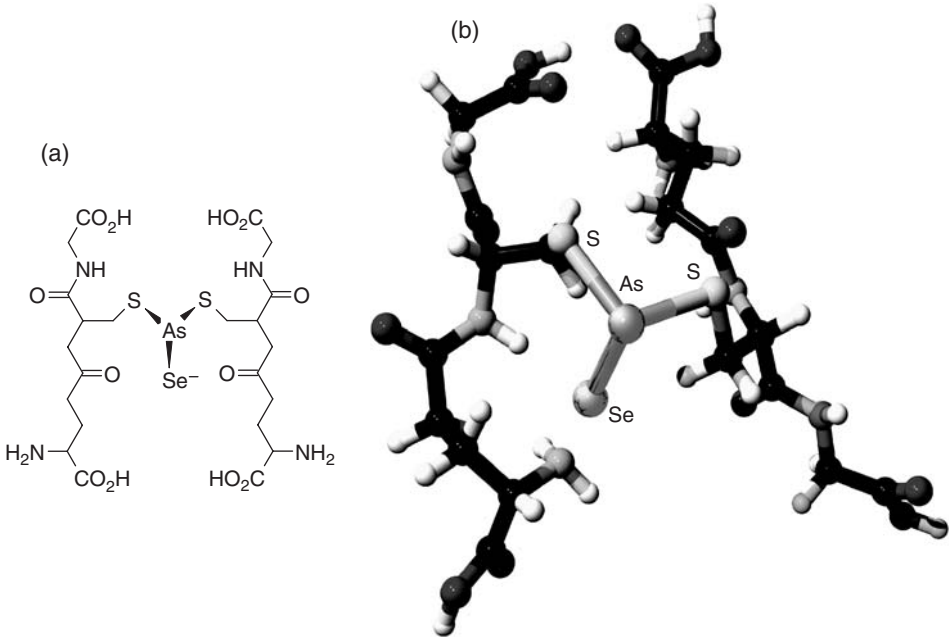


Figure 8 Structure of seleno-bis(*S*-glutathionyl)arsinium ion. (a) shows the schematic structure, while (b) shows the calculated structure of one conformer.

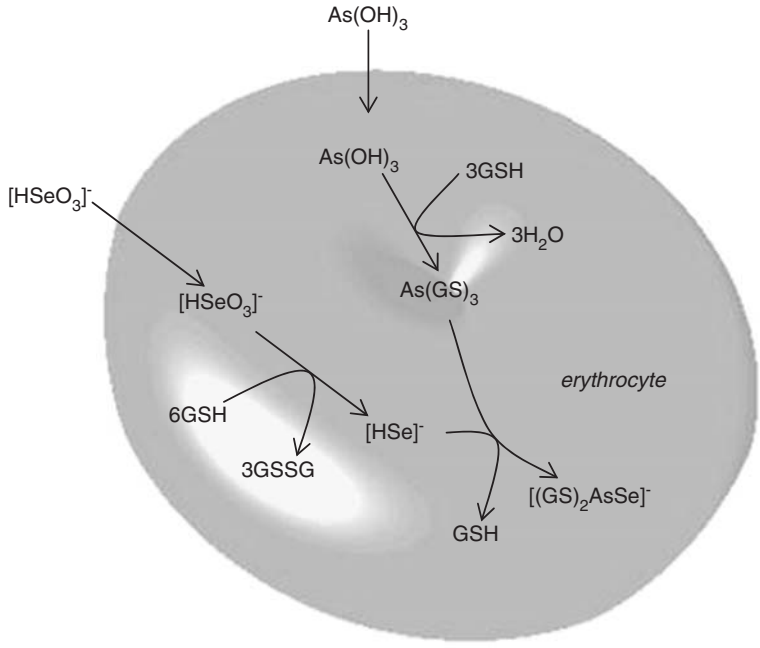


Figure 9 Postulated mechanism of formation of the seleno-bis(*S*-glutathionyl)arsinium ion.

The formation of the seleno-bis (*S*-glutathionyl)arsinium ion is thought to be a mechanism by which the body eliminates arsenic that necessarily involves the loss of one atom of essential selenium for every atom of arsenic excreted. This notion may have significance for the widespread poisoning of rural communities in Bangladesh and surrounding areas. Here, tens of millions of people are affected by a chronic low-level arsenic poisoning (arsenicosis) due to exposure to low levels (ca. 1 ppm) of arsenic in drinking water [53]. It has been suggested that the problem may be compounded because the contaminated water supply is also used to irrigate rice crops, which may take up and accumulate the arsenic, adding to the dose ingested by the population [54,55], although this idea has recently been criticized as representing only a minor component of the total arsenic intake [56]. The primary symptoms of the resulting chronic low-level arsenicosis are development of skin lesions, skin cancers, and finally internal tumors leading to death. Despite the magnitude of the problem (it has been called the worst mass poisoning in history), the biochemical mechanism by which arsenic causes these symptoms remains uncertain. The mystery deepens when one considers that there are communities elsewhere [57,58] that have similar or even higher arsenic in their drinking water, but no arsenicosis in the population. We have previously suggested that chronic low-level arsenicosis found in Bangladesh and elsewhere might in fact be an arsenic-induced selenium deficiency [40] caused by the formation and excretion of the seleno-bis(*S*-glutathionyl) arsinium ion. It is clear that selenium deficiency can cause cancer, and moreover, the diets of affected communities in Bangladesh are naturally low in selenium [59]. Conversely, the high arsenic communities exhibiting no arsenicosis have naturally higher dietary selenium. Selenium supplements have been suggested as a treatment [40], and two independent clinical trials involving administration of selenite [60] and selenomethionine [61] are currently underway.

5.2. Arsenic–selenium synergism

As we have discussed in the previous section, the antagonistic relationship between arsenic and selenium is now relatively well understood. There is, however, evidence for a poorly understood but pronounced synergistic toxicity between arsenite and methylated selenium metabolites [62,63]. When arsenite is administered to rats at a normally non-toxic dose followed by methylated selenium derivatives, again at normally non-toxic doses, then a significant toxic response is observed. The mechanism for this synergistic response is unknown, but has been suggested to be an inhibition of some (unknown) component of the detoxification pathway by arsenite [62]. We note in passing that in insects preliminary experiments appear to indicate no significant antagonistic response, but instead a synergism resulting in 100% mortality when both arsenic and selenium are given [64].

5.3. Mercury and selenium

Mercuric ions and selenite exhibit an antagonistic response that is not directly related to that observed with arsenic and selenium. In this case, reduction of

selenite by the reducing cellular environment is thought to form $[HSe]^-$, which reacts chemically with Hg^{2+} (probably bound to serum albumins), forming an inorganic mercury–selenium complex that contains approximately 100 atoms each of Hg and Se. We have used XAS to reveal that the complex is actually nano-particulate mercuric selenide [65], probably with a core zincblende structure, although the alternative wurtzite structure could not be excluded [65]. These nano-particles (Figure 10) subsequently bind to selenoprotein P, are carried in the blood plasma as an essentially inert species, and (probably) then stored in tissues as HgSe deposits. HgSe formation may be a major Hg detoxification mechanism in whales, which are exposed to large quantities of dietary Hg, and crystalline mercuric selenide (tiemannite) has been identified in whale liver [66]. Methylmercury species also show an antagonism with selenite [67], but in this case nanoparticulates do not form, and the mechanism for this is substantially unknown.

Perhaps even more intriguing than the antagonism discussed above is that instead of protecting, selenite can actually make matters worse when it is pre-administered to rats [68]. Under these circumstances, selenite is presumably

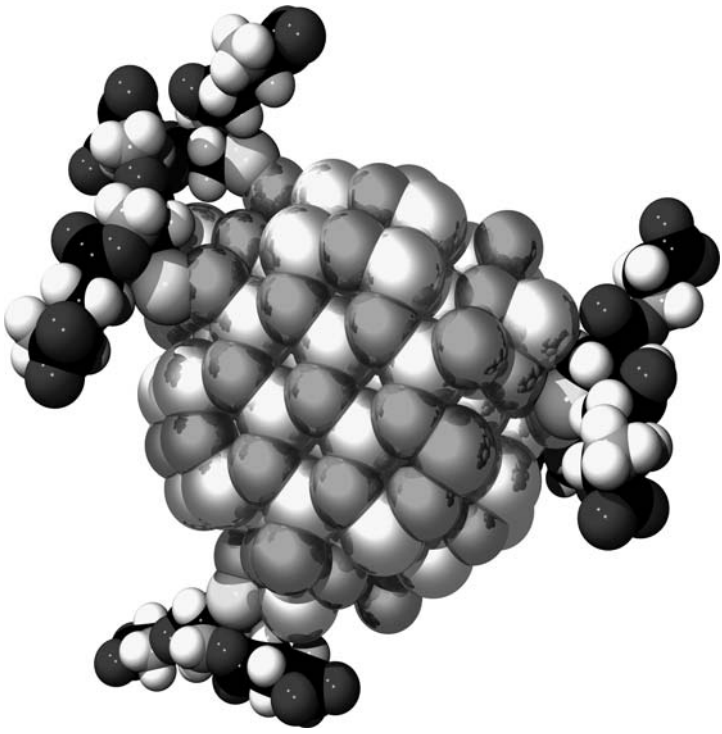


Figure 10 Molecular model of nanoparticulate mercuric selenide formed during the mutual detoxification of Hg^{2+} and selenite. The mercury and selenium are shown as dark and light metallic atoms, respectively, with glutathione moieties as external ligands to the HgSe core.

biotransformed, most likely being methylated to form dimethylselenide. This modified selenium then interacts (either directly or indirectly) with the mercury to form a more devastatingly toxic response. It is tempting to speculate that, while the As–Se and Hg–Se antagonisms (which we have discussed) are clearly distinct, the two synergisms are somehow related. As mentioned above, it has been suggested that arsenite inhibits some enzyme vital for the detoxification of methylated selenium species. Both arsenite and mercuric species have high affinity for thiols, so this is clearly a possibility but definitive proof must await further work. The similarly curious finding that selenomethionine protects adult mallard ducks against methylmercury chloride, yet increases its toxicity to ducklings [69] may also be related to the complicated and poorly understood interrelationship involving synergism and antagonism of mercury and selenium. Unlike arsenic, selenium and mercury show an antagonistic relationship in insects that appears to be quite similar to that found in mammals [70], which might argue against a common mechanism for the synergy.

5.4. Arsenic, mercury, and selenium

The extent to which these complex interrelationships can build is demonstrated by the three-way antagonism that has been reported for arsenic, mercury, and selenium with Japanese quail [71]. As expected from the known antagonistic relationship with selenium, survival rates of birds fed methylmercuric chloride dramatically increased when selenite was also fed to the birds (5–76% survival after 16 weeks). Significantly, survival increased to 100% when arsenite was fed together with both methylmercury chloride and selenite [71]. This three-way antagonism might be rationalized by formation of an excretible molecular entity involving all three elements. As we have discussed, $[(GS)_2AsSe]^-$ is formed in erythrocytes [41], and since methylmercury species can cross the erythrocyte membrane [72], it is chemically feasible that $[(GS)_2AsSeHgCH_3]$ could be formed in erythrocytes [73]. Practically, a triple poisoning involving arsenic, selenium, and mercury would be a most unlikely, not to mention unlucky, event, so the toxicology of this phenomenon is probably only of academic interest.

6. Chelation Therapy — Towards a Rational Design of Custom Chelators

Treatment of heavy metal or metalloid poisoning in humans often involves the use of chelation therapy. A chelator is a molecule that binds a metal or metalloid ion by at least two functional groups to form a stable ring-complex known as a chelate [74]. Chelation therapy is the clinical treatment by which heavy metals or metalloids are removed from or relocated within the body through binding to a chelation therapeutic drug to form a chelate, which is in most cases subsequently excreted in urine. One of the earliest chelation drugs was British anti-Lewisite (BAL) or 2,3-dimercaptopropanol (Figure 11) [75], which was

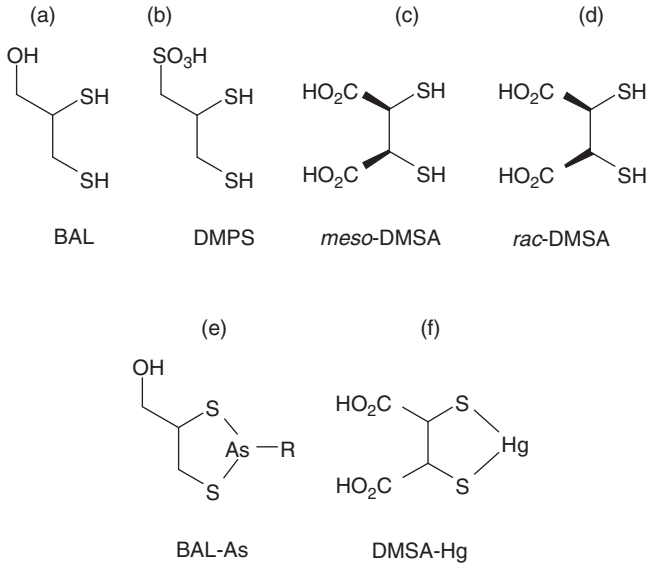


Figure 11 Structures of (a) British anti-lewisite, (b) dimercaptopropanesulfonic acid (DMPS), (c) meso-dimercaptosuccinic acid (*meso*-DMSA), (d) *rac*-DMSA, (e) As^{3+} chelate with BAL and (f) structure previously proposed for DMSA chelate with Hg^{2+}.

developed as an antidote for the arsenical war gas Lewisite (chlorovinylarsine-dichloride). Trivalent arsenicals such as Lewisite owe their short-term lethality to their strong binding to reduced dithiol of lipoic acid in the pyruvate dehydrogenase complex (and related enzymes) to make a six-membered ring. BAL will bind to arsenic to form the more stable five-membered ring (using DFT, we calculate a stabilization energy of 0.11 eV). BAL suffers from the disadvantages of low water solubility and a noxious smell, and so it was modified to produce dimercaptopropanesulfonic acid (DMPS) (Figure 11), which is water soluble and nearly odorless. Dimercaptosuccinic acid (DMSA) (Figure 11) was introduced later and has similar properties (i.e., excellent water solubility and no odor) with lower toxicity. The clinical use of DMSA differs from DMPS (and BAL) in that it is usually administered by mouth, whereas the others are generally given intravenously. DMSA and DMPS are commercially sold as Chemet® and Dimaval®, respectively, and both are used in treatment of heavy metal poisoning. However, as demonstrated below, while they are optimized for arsenic, their use with certain other metals may be far from ideal.

6.1. Mercury chelation therapy

A combination of XAS, chromatography and DFT has been used to characterize the binding of DMSA and DMPS by Hg^{2+} [76]. These studies showed that neither drug bound mercury as a chelator, forming the ring-shaped species

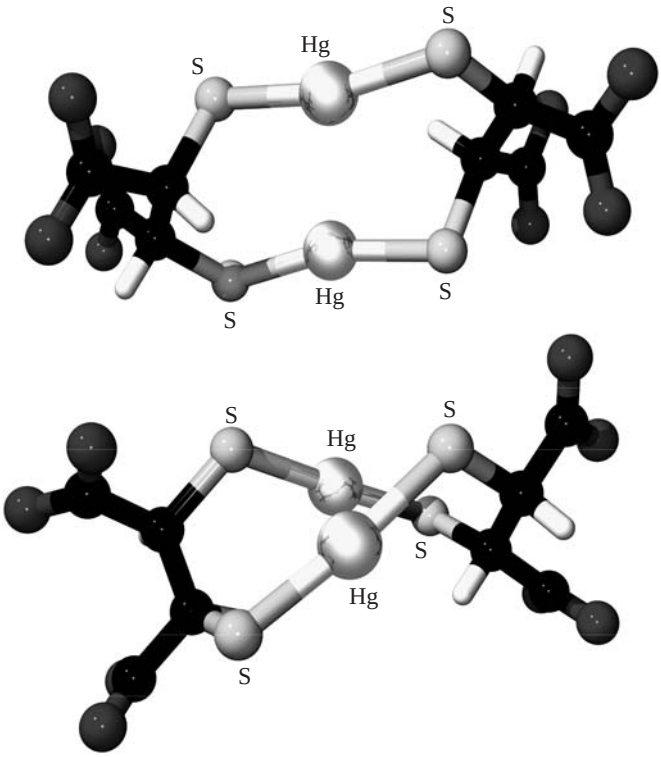


Figure 12 Calculated structures of the two diasteriomers of the smallest possible DMSA:Hg^{2+} complex. Carbon atoms are depicted as being black, oxygen as gray, hydrogen as white, mercury as metallic, and sulfur as light gray.

such as that shown in Figures 12 and 13 in which the drugs in fact act as mono-thiols, each donating one sulfur to the metal. DMSA and DMPS differ in that DMPS forms complexes with excess DMPS in which four DMPS molecules are ligated to mercury, each *via* a single sulfur, as shown in Figure 13b. No such four-coordinate species is formed with DMSA, and the most likely reason for this is that at physiological pH, the molecule would bear too large a negative charge to be stable. For example, a species with four DMSA molecules coordinating a single mercuric ion (as monofunctional reagents) analogous to Figure 13b, would bear the considerable negative charge of 14^-. Furthermore, the negatively charged carboxylate groups are much closer to the coordinating thiolate group than is the sulfonate of DMPS, and this proximity would further destabilize the complex. In agreement with this, DFT calculations predict stability of uncharged (protonated), but not of the charged species; in the charged case the ligands to the metal dissociate during the geometry optimization. DMPS, on the contrary, which bears only a single negative charge, can form stable four-coordinate species, and this is evident both from the experimental data and from DFT calculations [76].

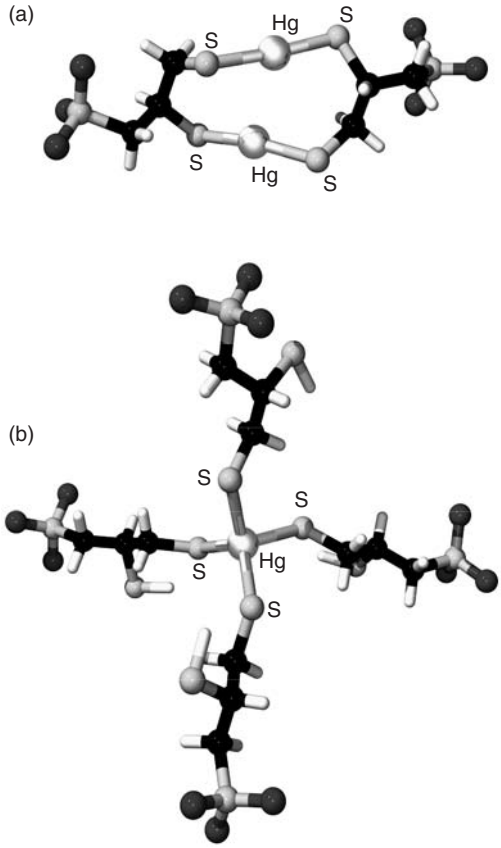


Figure 13 Calculated structures of possible Hg^{2+} — DMPS complexes. (a) Shows one of the four possible diasteriomers for linear Hg coordination and (b) shows the 4:1 complex that forms in the presence of excess DMPS.

Thus, despite their relatively widespread clinical use, DMSA and DMPS are actually not mercury chelators at all [76]. Furthermore, they are poorly optimized for this clinical role as they only act as mono-functional thiols in their interaction with mercuric ions, and the same is true for all of the agents currently used in mercury chelation therapy (i.e., none of them are actually mercury chelators). Having said this, there is no doubt that DMSA and DMPS are effective, to some degree, in the clinical treatment of mercury poisoning [77–81], although not in all cases [82]. Nevertheless, the fact that no mercury chelation drugs are in use means that were an actual chelator to be used then very considerably improved treatments should be possible. Furthermore, using tools such as DFT, it should be possible to design a chelator which would preferentially bind Hg^{2+} above other metal ions with similar coordination chemistries (e.g., Cu^{+}). We now discuss the criteria for the rational design of such a custom chelator.

One criterion for an effective drug for chelation therapy is that the chelator should interact in a stoichiometric manner with its target metal. In other words, if a single chelation drug molecule encounters a single target metal ion *in vivo* then these two species alone should be sufficient to form the chelate complex, with no need for additional species. We have shown that this criterion is not fulfilled by the clinically used mercury chelation therapeutic drugs, DMSA and DMPS (Chemet® and Dimaval®) [76] which need at least 2 drug molecules per mercury. Here we will consider two possible geometries for such a custom chelator — linear two-coordinate and trigonal planar.

A custom chelator optimized for digonal coordination should present the metal with two thiol groups whose sulfurs are separated by twice the optimal Hg–S distance (i.e., 2×2.345 Å). By comparing the calculated energetic dependence of parameters such as the Hg–S bond-length, the C–S–Hg and S–Hg–S bond-angles, and the C–SHgS–C torsion angle, the structural factors that are important in binding Hg^{2+} can be evaluated. Thus, we have found that precise control of the Hg–S bond-length, the C–S–Hg bond-angle and the C–SHgS–C torsion angle is important, but not so for the S–Hg–S bond-angle which can deviate by almost 10° without significant penalty [76]. A chelator molecule with donors rigidly held by a molecular framework in these positions would bind mercury with high affinity, and we calculate the order of 10^6 fold higher than mono-functional agents such as DMSA or DMPS. Such custom chelator molecules would have inherent metal ion specificity, which is primarily conferred from the combination coordination geometry and bond-length. For example, numerous zinc thiolate species have been characterized, with bond-lengths of about 2.35 Å (i.e., approximately the same as the two-coordinate Hg–S bond-length), but with a tetrahedral coordination geometry. As we have mentioned above cuprous ions readily adopt a linear two-coordinate coordination geometry, but the much shorter Cu–S bond-length of around 2.15 Å means that a mercury custom chelator would be too big to accommodate cuprous ions. The next step in the development of a custom chelator molecule is the design of an organic framework that can hold the ligand groups in the desired geometry. While this is clearly a non-trivial matter non-rigid candidates are straightforward to produce.

The ideal chelator would have a binding site that is accessible to the target metal ion, would have no biological activity in its own right, and would be stable enough to be handled in air and at room temperature. The first consideration effectively excludes tetrahedral dispositions of ligands, while the latter effectively excludes molecules with selenide donors. Other critically important properties, such as water solubility and the ability to cross cell membranes might be adjusted by altering the organic framework holding the metal donor atoms. Probably the ideal arrangement for mercury is a three-coordinate trigonal planar system with thiolate ligands to the metal. This combines stability with an accessible binding site, and it is probably not a coincidence that this geometry is adopted in nature by MerR, the metalloregulatory protein of the mercury-resistance operon which has an apparent affinity for Hg^{2+} of $\sim 10^{-8}$ M in the presence of millimolar levels of competing thiols [83]. DFT calculations indicate that three-coordinate Hg–S has an ideal bond-length 2.454 Å, with Hg–S–C bond-angles of 102.8° and C3 symmetry.

Known trithiols such as 2-[3,5-bis-(2-mercapto-ethyl)-phenyl]-ethanethiol [84] can certainly potentially coordinate mercury with close to the desired geometry, as can the more rigid *S*-cylindrophanes [85], although we note that these last present thioether ligands to the metal, which will bind less effectively than thiolates. One candidate that has yet to be synthesized, and which displays many of the properties which we seek, is the cylindro-tri-thiol (1,3,5-tris-3-mercapto-hexyl-bis-phenyl) shown in Figure 14. These compounds will have limited water solubility and modification by substitution of carboxylic acid or sulfonate groups would be needed to create a practical chelator.

We note that there are many additional factors in the design of an effective drug that we have not considered here, such as inherent toxicity of the custom chelator itself, and how readily the chelate (once formed) is excreted. These factors mean that extensive studies upon the clinical effectiveness of any new drug will be required. Furthermore, effective metal chelation does not necessarily imply effective treatment of heavy metal poisoning. For example, in the case of Tl^{+} poisoning, chelation therapy with diethyldithiocarbamate (variously called Dithiocarb, Imuthiol or DETC, in the clinical setting) [86] causes increased excretion of thallium in the urine, but also increases mobilization of Tl^{+} to the central nervous system, with considerable resulting adverse effects. This tendancy to mobilize the metal to the brain has been attributed to the lipophilic nature of the

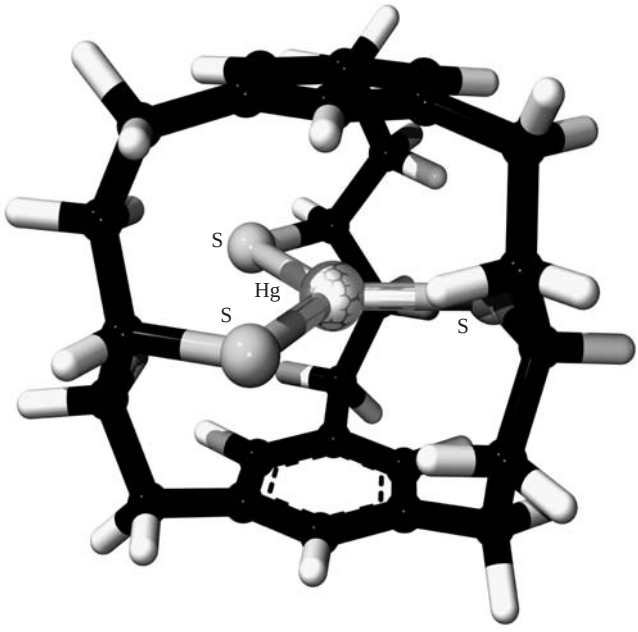


Figure 14 Structure of a possible custom chelator for Hg^{2+} based on trigonal planar thiolate coordination. This chelate is merely an example of the type of molecule which might be used, and is not a serious candidate for a custom chelator. More optimal structures might incorporate thiols rigidly held in their ideal positions.

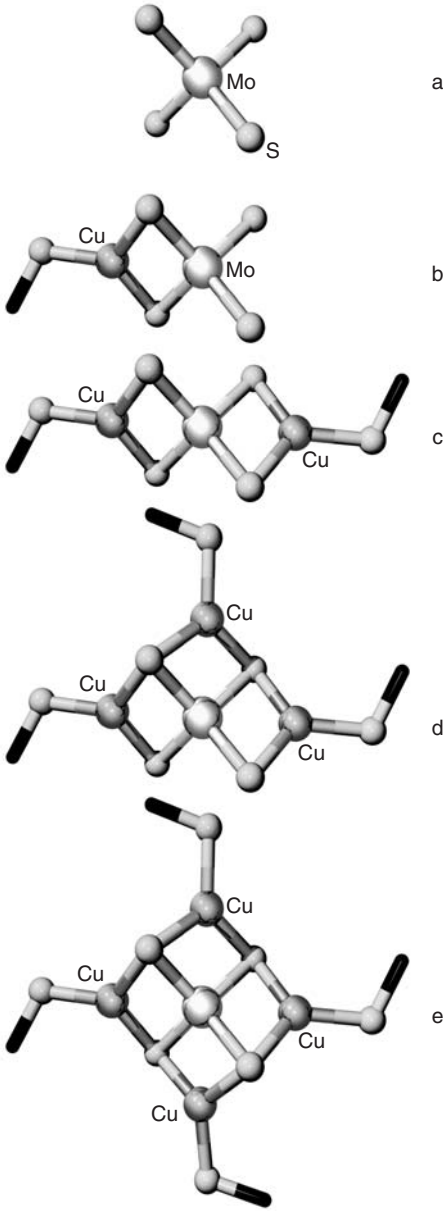


Figure 15 Thiomolybdate and its clusters with cuprous ions. a shows the tetrahedral thiomolybdate anion $[MoS_4]^{2-}$, and complexes with one through four cuprous ions are shown in b–e.

thallium diethyldithiocarbamate chelate. The use of diethyldithiocarbamate with thallium poisoning is thus contra-indicated [87], but in an interesting side-note copper diethyldithiocarbamate complexes have been suggested as a possible treatment for Menke's disease (a disease of copper deficiency, related to Wilson's disease), and some success in an animal model has been reported [88].

6.2. A novel chelation approach — Wilson's disease and thiomolybdate

An example of a potentially successful chelation therapy is given by a new approach to the treatment of Wilson's disease. Wilson's disease is an autosomal recessive hereditary disease, with an incidence of about 1 in 30,000 in most parts of the world, and a male preponderance. Its main feature is accumulation of copper in tissues, which manifests itself with neurological symptoms and liver disease [89]. In healthy individuals copper is exported from the cell by "packaging" in the Golgi body *via* a specific ATPase copper-transport protein (the Wilson's protein). Wilson's disease sufferers are deficient in this protein due to a mutation in the ATP7B gene, and copper accumulates in the body, particularly in the liver. A convenient animal model of Wilson's disease is provided the Long Evans Cinnamon (LEC) rat. Dietary thiomolybdate has already been used in humans to block copper uptake from gut, but interperitoneal injection of tetrathiomolybdate in LEC rats has a much greater effect [90]. Tetrathiomolybdate injection prior to the onset of hepatitis and of neurological symptoms protects the rats from these effects. Injection at later stages (e.g., after advanced neurological problems have developed) restores rats to good health within $\sim$24 h. Tetrathiomolybdate can form adducts with cuprous ions by bridging across two Mo = S groups to form multi-metallic clusters, and the structures of four such clusters are shown in Figure 15. The formation of similar clusters is thought to be responsible for the molybdenum-induced copper deficiency that occurs in ruminants feeding on a molybdenum rich diet [91]. In this case molybdates in the diet are thought to be converted to thiomolybdate in the rumen, which binds copper, preventing absorption and casing copper deficiency. Returning to LEC rats, examination of liver lysosomes prepared from LEC rats using XAS studies at both the Cu K- and Mo K-edges indicated that novel chelation complexes form in the liver [92]. These predominantly had a $[Cu_3MoS_4]^+$ core (Figure 15d), which is the most common of these clusters described in the Cambridge crystal structure database [17].

7. Conclusions and Future Directions

The increasing availability and sensitivity of *in situ* advanced spectroscopic probes such as XAS is expected to support important advances in molecular toxicology. The use of advanced quantum mechanical codes in combination with *in situ* spectroscopic probes has significant advantages in providing a reality check. The models that are constructed to accurately describe the biochemistry of toxic

metals must be as rigorous, at all levels, as the limits of our applied techniques. Furthermore, it is imperative that a chemically accurate description of metals or metalloids and their ligands be retained as a central premise of any such model.

Design of custom chelators is expected to be important in therapy of heavy metal poisoning. The specificity of such chelation drugs will be especially important as exemplified by recent deaths from hypocalcemia with EDTA (ethylenediaminetetraacetic acid) chelation therapy [93]. Finally, heavy metal coordination represents a research opportunity for coordination chemists. For example, plutonium exposure is treated with DTPA (diethylenetriaminepentaactetic acid) and a number of other chelators [94], but no structural information on these complexes is published, and the mode of binding of the chelator is thus unknown.

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CHAPTER 6

Pyrimidine Damage and Repair

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Contents

Abstract

Pyrimidines are essential to the structure and function of nucleic acids in all organisms. The chemical reactivity of the pyrimidines is therefore a fundamentally important aspect

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of survival of all organisms. Pyrimidines are subject to endogenous damage through hydrolysis, alkylation and oxidation, and to damage by exogenous environmental molecules. Damage to these important molecules can result in loss of biochemical function and cell toxicity. The damage of pyrimidines in deoxyribonucleic acids (DNA) could have more long-lasting consequences, as many of the pyrimidine lesions are miscoding during DNA replication resulting genetic mutations, and allowing the effects of the damage to be transmitted to future progeny cells. Emerging evidence also suggests that pyrimidines in DNA play a critical role in establishing transcriptional regulation in complex organisms, and that damage to DNA pyrimidines could alter epigenetic programming. Fortunately, repair systems exist in cells that recognize specific damaged structures and facilitate their removal and ultimately DNA repair.

1. Biologically Important Pyrimidines

The nucleic acids of most organisms contain uridine and cytidine in ribonucleic acids (RNA) and 2′-deoxycytidine and thymidine in deoxyribonucleic acids (DNA). In the DNA of many higher organisms, a few percent of the 2′-deoxycytidine residues are replaced by 5-methyl-2′-deoxycytidine (Figure 1).

In biosynthetic pathways common to most organisms, pyrimidine synthesis begins with carbamoyl phosphate. Addition of the amino acid aspartate followed by an intramolecular ring closure and dehydration yields orotic acid. In a complex of proteins, orotic acid is coupled with a ribose sugar phosphate, and the

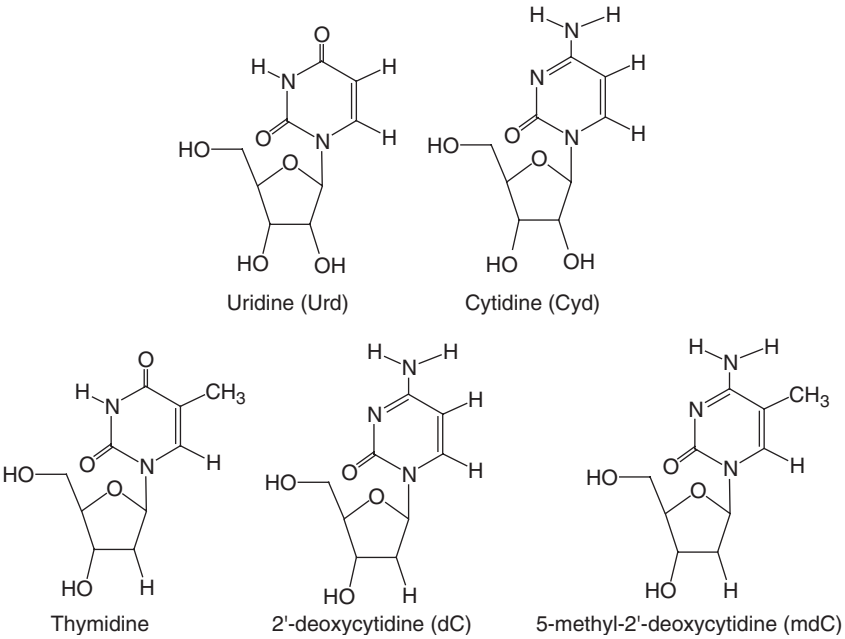

Figure 1 Structures of uridine, cytidine, dC, thymidine and 5-methyldC.

6-carboxy group is removed generating uridine monophosphate (UMP). UMP is the precursor for all other pyrimidines. An amino group from glutamine is donated, converting the uridine to cytidine analogs. Both uridine and cytidine can be enzymatically converted to the corresponding 5′-mono, di and triphosphates [1].

The corresponding triphosphates are precursors for RNA polymerase-directed RNA synthesis. Ribonucleotide triphosphates can also be enzymatically hydrolyzed to the corresponding mono- and diphosphates. Both cytidine and uridine diphosphates are converted to the corresponding 2′-deoxynucleotides by ribonucleotide reductase, an iron-centered free-radical generating enzyme [2]. Deoxycytidine diphosphate is enzymatically converted to the corresponding triphosphate where it can serve as a substrate for DNA polymerase. Deoxyuridine diphosphate can also be converted to the triphosphate where it can serve as a substrate for DNA polymerase, in place of thymidine triphosphate [3]. However, uracil is not a normal component of DNA and it is recognized as a form of damage and is removed by a series of repair enzymes discussed further below.

The generation of thymidine triphosphate involves additional steps. Deoxyuridine diphosphate, a product of ribonucleotide reductase, is enzymatically hydrolyzed to 2′-deoxyuridine 5′-monophosphate or dUMP. The difference between thymidine monophosphate (TMP) and dUMP is the methyl group in the 5-position of the ring in TMP (see Figure 1). The enzyme that carries out this transition is thymidylate synthase, utilizing methylene tetrahydrofolate as the methyl donor [4,5]. TMP can be converted to the corresponding di- and triphosphate where it serves as a substrate for DNA polymerase.

An understanding of the biosynthetic pathways leading to nucleotides and deoxynucleotides is important for the introduction of both stable and radioactive isotopes often utilized in nucleic acid studies. Furthermore, numerous nucleoside analogs and other drugs that interfere with nucleotide metabolism are potent antiviral and antitumor compounds [6–11]. Major drug targets are ribonucleotide reductase, inhibited by hydroxyurea, and thymidylate synthase, targeted by 5-fluorouracil after conversion to the corresponding deoxynucleotide monophosphate. The formation of methylene tetrahydrofolate can be inhibited by drugs such as methotrexate, which is a potent antiproliferative agent in man. Sulfa drugs, which inhibit folate synthesis in bacteria, are potent antibacterial agents. In man, deficiencies in the vitamins folic acid or vitamin B12 can result in deficiencies of methylene tetrahydrofolate, resulting in reduced cell proliferation. Also, in man, nutrient deficiency can result in anemias, whereas in the developing fetus, folate deficiency can cause dramatic malformations including neural tube defects, such as spina bifida [12–16].

2. Physical Properties of the Pyrimidines

Many of the physical properties of the pyrimidines are essential in determining their biological properties. The physical properties are also central to many of the analytical tools used in the study of pyrimidines and nucleic acids. The solubility of

the pyrimidine free bases in water ranges from 30 to 350 g/100 ml at 25°C [17,18]. Surprisingly, the 5-methyl pyrimidines, thymine and 5-methylcytosine are more water soluble than the unmethylated analogs, by factors of 2–10. The enhanced water solubility of the 5-methyl pyrimidines has been attributed to increased induced-dipole (pyrimidine)-dipole (water) interactions [18,19]. The nucleoside and nucleotide analogs of the pyrimidines are substantially more water soluble.

The water solubility of the pyrimidines, as well as their characteristic ultraviolet (UV) spectra renders UV spectrometry one of the most valuable laboratory techniques in studies with pyrimidines and their analogs. Most pyrimidine derivatives absorb well in the UV spectrum. The absorbance maxima of the normal, neutral pyrimidines range from 260 to 275 nm with molar absorption coefficients approximately 1×10^4. The spectral properties can be invaluable in identification and quantitation of pyrimidines in aqueous solution [20,21]. The UV absorbance maxima of the 5-methyl pyrimidines is approximately 5 nm to longer wavelengths relative to the unsubstituted pyrimidines.

In aqueous solution, the pyrimidines can undergo ionization (Figure 2). The uracil analogs ionize with pK_a values approximately 9.5, whereas the cytosine analogs undergo protonation with pK_a values approximately 4.5 [22]. The ionization of the pyrimidines as a function of solution pH is easily monitored by measuring the pH-dependence of the UV spectrum. Modifications of the pyrimidines can induce profound changes in pK_a values. Several pyrimidine derivatives of biological interest have substituents in the 5-position. Electron donating substituents tend to shift pK_a values higher, whereas electron withdrawing substituents shift pK_a values lower [22–25].

Linear relationships have been established for the pK_a values of 5-substituted pyrimidines as a function of the Hammett meta parameter (σ_m) for

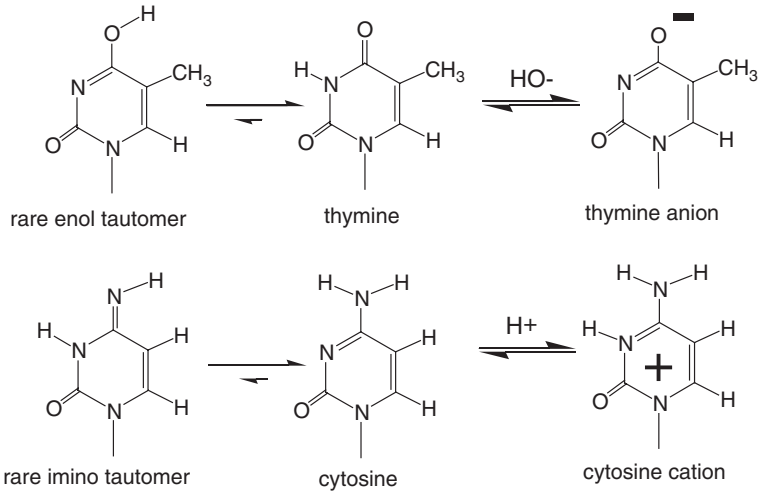

Figure 2 Ionization and tautomerization of the pyrimidines.

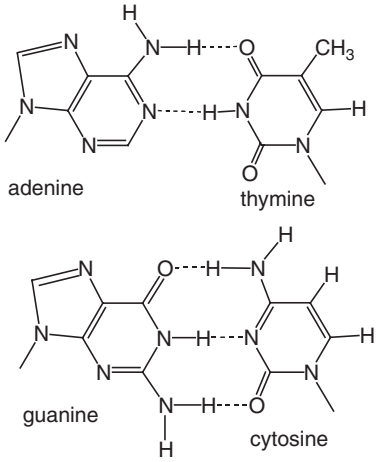

Figure 3 Watson–Crick base pairs.

the 5-substituent [23–25]:

5-substituted 2′-deoxyuridine analogs: $pK_a = 9.47 + (-4.34)(\sigma_m) \pm 0.14$
5-substituted 2′-deoxycytidine analogs: $pK_a = 4.09 + (-3.98)(\sigma_m) \pm 0.19$

The neutral pyrimidines assume the keto and amino tautomeric forms in aqueous solution. Protons can shift from one heteroatom to another, generating rare enol or imino tautomers (Figure 2). Historically, the rare tautomeric forms of the pyrimidines have been invoked to explain miscoding during polymerase-directed DNA replication [26]. The pyrimidines assume the rare tautomeric forms with frequencies of approximately 1 in 10^5 [27,28].

One of the most profound properties of the pyrimidine derivatives is the capacity to form specific hydrogen bonds when in polynucleotide structures. In Figure 3, the structures of the canonical Watson–Crick base pairs are shown. The formation of these base pairs is essential to the correct heritable transmission of genetic information in all organisms [26]. The reliance upon specific hydrogen bond formation reveals that modifications of the pyrimidines that might alter the position of protons on specific heteroatoms could have a profound effect upon the capacity to form correct or incorrect base pairs. Upon the basis of this important role of specific hydrogen bond formation, substantial efforts have been invested in understanding how chemical modifications of the pyrimidines might alter proton configurations, thus reducing the fidelity of DNA replication, and increasing the chances of base mispair formation and mutagenesis.

3. Hydrolytic Damage

3.1. Glycosidic bond cleavage

The nucleic acids of most organisms are within an aqueous environment for most of the life cycle. Pyrimidines in nucleic acids are subject to hydrolysis reactions

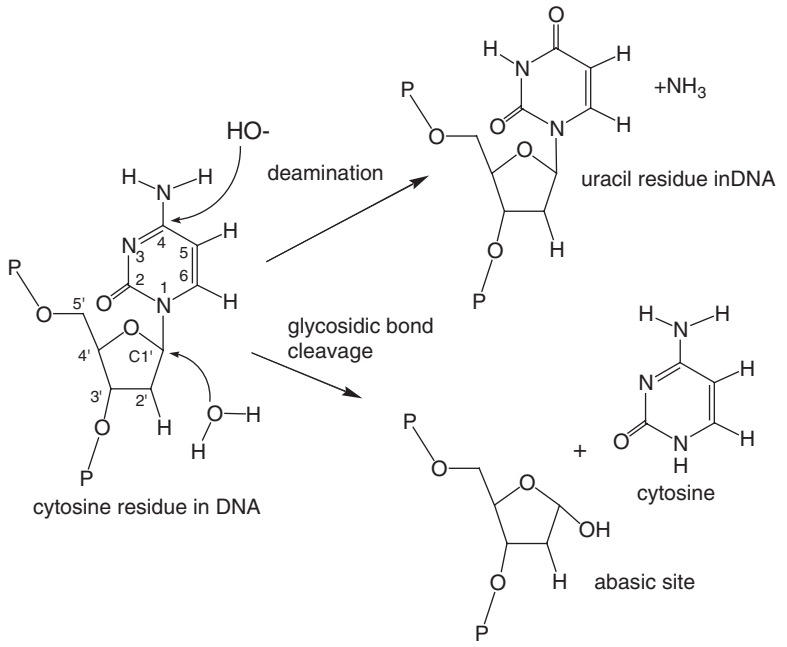

Figure 4 Glycosidic bond cleavage and deamination.

including both cleavage of the glycosidic bond and deamination [29–35], and thus water molecules constantly attack the nucleic acids (Figure 4).

As shown in Figure 4, water can attack the C1′ position of the sugar, resulting in displacement of the pyrimidine moiety. The biologically important reaction product is the abasic site remaining attached to the phosphate backbone of the polynucleotide structure. In general, the glycosidic linkage of the 2′-deoxynucleotides is thousand times more labile than the corresponding linkage of the ribonucleotides, and the pyrimdines are more stable than the corresponding purine analogs to hydrolysis. The 2′ hydroxyl group found in ribonucleotides deactivates the C1′ position from nucleophilic attack by water. Substituents in the pyrimidine ring can also have a profound effect upon the rate of glycosidic bond cleavage. Electron-donating substituents in the 5-position, including a methyl group, stabilize the glycosidic bond whereas electron-withdrawing substituents, such as halogens, increase hydrolysis rates.

The hydrolytic reactions of the normally occurring 2′-deoxynucleosides are generally too slow to measure at physiological temperature. Rates are experimentally measured at higher temperatures and extrapolated to physiological conditions. The measured rates and activation energies reported by different laboratories are sufficiently close to estimate the rates of these events per cell per day under physiological conditions.

The rate constant for hydrolysis of the glycosidic bond of 2′-deoxycytidine in solution has been estimated to be $1 \times 10^{-13}\,s^{-1}$ at 37°C at neutral pH with a corresponding activation energy of approximately 40 kcal/mol [31,35]. When

placed within a duplex polynucleotide structure, the rate of reactivity is reduced by approximately one order of magnitude. The corresponding half-life for loss of cytosine from duplex DNA by spontaneous hydrolysis of the glycosidic bond would then be approximately 22,000 years. As the rates of glycosidic bond cleavage for thymidine and 2′-deoxycytidine are similar in magnitude, it can be estimated that approximately 20–30 pyrimidines are lost from the human genome per cell per day.

The biological consequence of glycosidic bond cleavage, with generation of an abasic site, is loss of genetic information. When a DNA polymerase would encounter the abasic site, the pyrimidine that would have directed the incorporation of either adenine or guanine through formation of a correct base pair has been lost, and there is no basis upon which to select the correct base for insertion [36]. The abasic site is an intermediate in the base-excision repair (BER) pathway as described in more detail below.

3.2. Deamination of cytosine analogs

As shown in Figure 4, water can attack the C4 position of the pyrimidine ring, converting the cytosine analog to the corresponding uracil analog. The biological consequence of this reaction is a change in coding properties. In undamaged DNA, cytosine directs the incorporation of guanine during polymerase-directed DNA replication through the formation of correct hydrogen bonds. However, hydrolysis of cytosine to the corresponding uracil analog would direct the incorporation of adenine rather than guanine. The corresponding change of guanine to adenine is referred to as a transition mutation [34].

The mechanism of deamination has been studied in solution. The apparent rate constant is approximately $1 \times 10^{-10}\,s^{-1}$ with an activation energy of approximately 30 kcal/mol [32,35]. The deamination reaction is accelerated by cytosine protonation. As the site of protonation is occupied by base pair formation in duplex DNA, the corresponding rate of deamination drops by a factor of 100 in duplex DNA [32]. Approximately 100–200 cytosine residues in the human genome would be converted to uracil per cell per day. If unrepaired, the resulting uracil would code like thymine, giving rise to a transition mutation.

The deamination of 5-methylcytosine has also been studied in aqueous solution [37–39]. In neutral or alkaline solution, 5mC analogs deaminate two to three times faster that the corresponding cytosine analogs, however, in acid solution, the cytosine analogs deaminate faster. The increase in the deamination rate for 5mC at physiological pH could be attributed to the greater basicity of 5mC and the higher frequency of protonated molecules, relative to cytosine [35]. Approximately 5% of cytosine residues are methylated in the human genome [40]. Therefore, approximately 10–20 5mC residues would deaminate to thymine per cell per day under normal physiologic conditions.

The deamination of 5-methylcytosine is a special case with profound biological importance. Whereas uracil, the deamination product of cytosine, is recognized by several very active glycosylases found in essentially all organisms, the deamination product of 5-methylcytosine is thymine. As thymine is a normal component of DNA, the identification and repair of thymine arising from 5-methylcytosine

deamination presents a special challenge for DNA surveillance and repair systems. In the human genome, 5-methylcytosine residues are found predominantly in the CpG dinucleotide. Transition mutations at CpG dinucleotides represent the most frequent single base change found in human cancer cells [41–43].

4. Oxidation Damage of Pyrimidines

4.1. Formation and reactions of pyrimidine glycols

In the process of normal cellular metabolism, electrons are transferred, ultimately to oxygen. In this process of biological oxidation, loose electrons can react with molecular oxygen, forming the reactive molecule, superoxide. Superoxide can be enzymatically converted to hydrogen peroxide by superoxide dismutase, which is then converted to water by catalase. The intermediate superoxide and hydrogen peroxide can be involved in oxidation chain reaction, ultimately reacting with nucleic acids, protein and lipids in membranes.

The most frequently formed oxidation damage product in DNA is thymine glycol [44–46], which requires two hydroxyl radicals (Figure 5). Thymine glycol is a non-planar pyrimidine analog, which has lost it aromaticity and UV absorbance

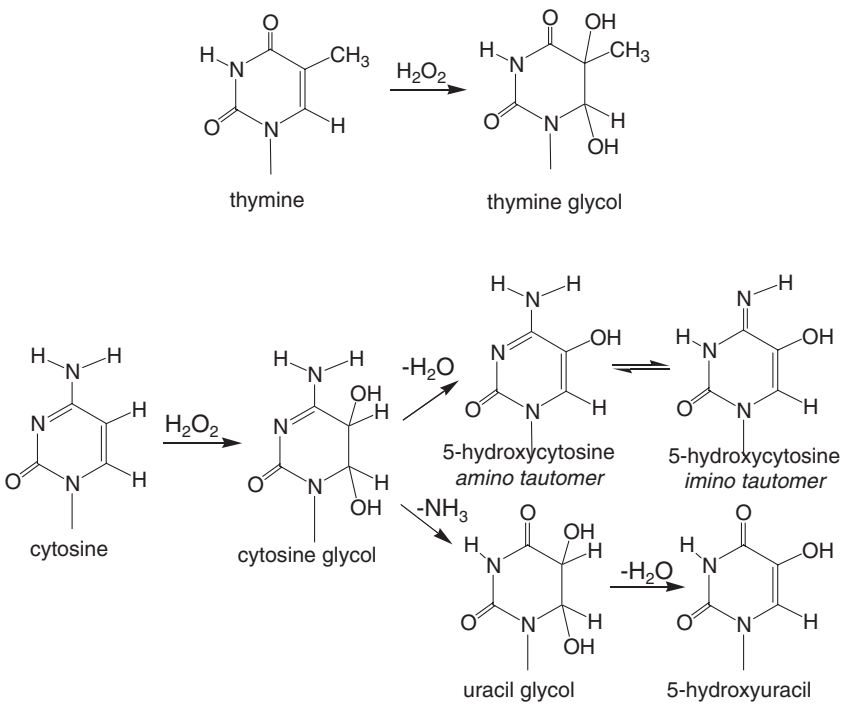

Figure 5 Formation of pyrimidine glycols.

near 265 nm. Although thymidine glycol can miscode, it primarily acts as a block to DNA polymerase [46]. The 5-methyl group is no longer in the plane of the base, which causes a steric clash with surrounding bases. Thymine glycol is subject to further hydrolysis, characteristic of the ring-saturated pyrimidines. Water can attack at the pyrimidine ring of saturated derivatives, resulting in a series of ring-opened structures. Glycols can also be generated with strong oxidizing chemicals including potassium permanganate and osmium tetroxide, agents used to selectively break DNA chains at thymine, or 5-methylcytosine residues [47–49]. Ring-saturated pyrimidines can also be generated by photohydration and pyrimidine dimer formation [50–51].

Cytosine analogs can similarly form glycols (Figure 5) [52]. Unlike the relatively stable thymine glycol, however, the cytosine glycols can undergo both deamination and dehydration. Deamination results in the formation of the corresponding uracil analogs. Dehydration can occur before or after deamination, and result in the formation of the 5-hydroxy analogs, 5-hydroxyuracil and 5-hydroxycytosine. Physical studies have shown that the 5-hydroxy group can form an intermolecular hydrogen bond with the 5-phosphate in nucleotide analogs [24]. The cytosine analog, 5-hydroxycytidine, has been shown to be one of the most mutagenic of all base analogs [53]. The miscoding potential of 5-hydroxycytosine has been attributed to an enhanced tendency to form the imino tautomeric form [54,55].

4.2. Oxidation of the thymine methyl group

In addition to reaction with the pyrimidine 5–6 bond, hydroxyl radicals can also abstract a hydrogen atom from the thymine methyl group [56–64]. Reaction with another hydroxyl radical then gives the chemically stable 5-hydroxymethyluracil (HmU) analog. HmU can be further oxidized to the 5-formyl and 5-carboxyl analogs that can further decarboxylate, forming uracil analogs [65] (Figure 6).

The conversion of thymine to HmU does not disrupt normal B-form DNA conformation [66] or induce miscoding during polymerase-directed DNA synthesis. Templates containing HmU are copied by both RNA and DNA polymerases, and HmU completely replaces thymine in some bacteriophages [67]. However, in higher organisms including humans, HmU residues in DNA are removed by BER. Indeed, sufficient numbers of HmU residues can be incorporated into the DNA of cells grown in 5-hydroxymethyl-2′-deoxyuridine to trigger apotosis or programmed cell death [56,57]. The apparent lethal repair of a non-coding lesion caused us to further consider potential detrimental consequences of HmU in DNA as discussed further below.

In contrast to HmU, the further oxidation damage product, 5-formyluracil (FoU) is very mutagenic, resulting in transition mutations [23,62–64,68]. The mutagenicity of FoU has been attributed to the effect of the extremely electron-withdrawing 5-formyl substituent on the pK of the N^3 proton [23]. Conversion of thymine to FoU results in a drop of the N^3 pK from above 9 to near 7, substantially increasing the proportion of the ionized base capable of mispairing with guanine. Polymerase studies conducted at several values of solution pH

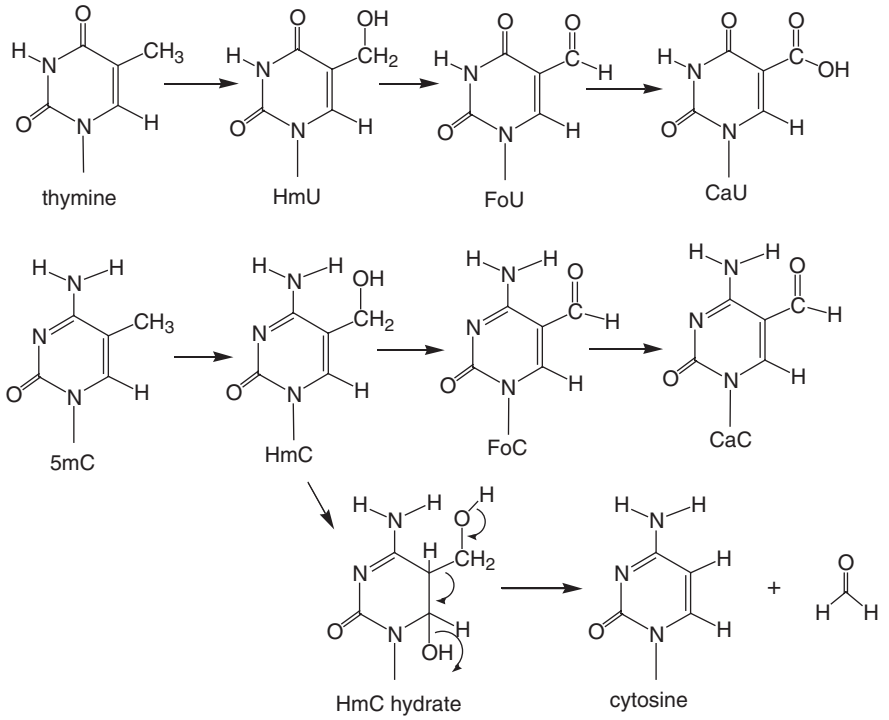

Figure 6 Oxidation of pyrimidine methyl groups.

support the concept that the miscoding of FoU is driven by ionization of the N^3 proton [68]. As with HmU, the presence of FoU can disrupt sequence-specific protein interactions. The 5-formyl group could also conceivable result in DNA–protein crosslinks. Although the free base and nucleoside analogs do react with amines [69], FoU residues in DNA appear to be sterically protected from reaction with amines [70]. Little is as yet known about the biological consequences of 5-carboxyuracil. However, the 5-carboxy substituent would be ionized at physiological, electron-donating to the pyrimidine ring, and inhibiting and opposed to facilitating ionization of the N^3 proton.

4.3. Oxidation of the methyl group of 5-methylcytosine

As with the methyl group of thymine, the methyl group of 5mC can be similarly oxidized [71,72], forming the 5-hydroxymethyl (HmC), 5-formyl (FoC) and 5-carboxy (CaC) analogs. In some bacteriophages, HmC replaces cytosine. None of the 5-oxidized cytosine analogs would be expected to have substantial miscoding properties. A glycosylase that selectively removes HmC has been identified in higher eucaryotes [73]. The methyl group of 5mC is slightly more reactive than that of thymine toward radical oxidation. Somewhat surprisingly, the relative

reactivity of methyl groups is influenced by surrounding base sequence by less than a factor of 2 [72].

The oxidation of the pyrimidine methyl groups can result from the presence of reactive oxygen species (ROS) and from UV radiation. In aqueous solution, 5mC can be converted to a number of analogs, including cytosine. While cytosine would result from the sequential oxidation of the 5-methyl group, photochemical oxidation of 5-carboxycytosine does not yield cytosine in high yield. However, upon UV irradiation, HmC is converted directly to cytosine with little formation of 5-carboxycytosine. It has been proposed [71] that the formation of the intermediate photohydrate of HmC could yield cytosine by elimination of the hydroxymethyl group (Figure 6). Indeed, early studies indicated that the storage of 5-hydroxymethylpyrimidines is alkali solution could result in substantial loss of the 5-hydroxymethyl group [74,75], probably through hydration of the 5–6 bond followed by elimination [76].

The saturation of the 5–6 bond of 5-hydroxymethylpyrimidines, followed by elimination could be important mechanistically in the action of enzymes that act to modify or repair DNA. In the biosynthesis of thymidine from 2′-deoxyuridine by thymidylate synthase using methylenetetrahydrofolate, the final step is an irreversible reduction, generating the thymidine methyl group. In some bacteriophages, hydroxymethyl derivatives of both 2′-deoxyuridine-5′-monophosphate and 2′-deoxycytidine-5′-phosphate are formed enzymatically using methylene tetrahydrofolate as the one-carbon donor by enzymes structurally similar to thymidylate synthase [77]. In contrast with thymidylate synthase, the enzymatic hydroxymethylase reactions are reversible. The reverse enzymatic reaction is similar to the elimination of hydroxymethyl group from ring-saturated pyrimidines. In solution, the hydroxymethyl derivatives can be saturated by photohydration or hydration, leading to elimination of the hydroxymethyl group, whereas the hydroxymethylases could act in an analogous manner to saturate transiently the 5–6 bond, leading to elimination of the 5-hydroxymethyl group. DNA methyltransferases, structurally similar to thymidylate synthase and the hydroxymethylases, also form a 5–6 ring saturated intermediate [78–80]. Recently, 5mC demethylase activities have been described [81,82] that act by direct displacement of the methyl group yielding methanol. This mechanism is chemically unlikely. However, the oxidation of 5-methylcytosine forming HmC, followed by ring saturation could be a plausible mechanism for the demethylation of 5mC in DNA [83], a suggestion strengthened by the recent identification of the mechanism of repair "demethylases" identified in higher eucaryotes and humans [84,85] that act *via* an oxidative mechanism.

4.4. The important role of the pyrimidine 5-methyl group in the DNA of higher eucaryotes

The 5-methyl groups of thymine and 5mC are found in the major groove of a B-form DNA helix. The methyl groups are on the opposing side from the hydrogen bonding face and do not participate in hydrogen bonding or base-pair formation. Methyl groups contribute slightly to the increased thermal stability of

oligonucleotides containing 5-methyl pyrimidines, thymine and 5mC, relative to unmethylated duplexes containing uracil and cytosine respectively [18]. The increased melting temperatures have been explained on the bases of the greater base-stacking of the methylated pyrimidines, resulting from increased molecular polarizability [18,19]. While pyrimidine methylation in the 5-position slightly enhances duplex stability, the important contribution of the pyrimidine 5-methyl group is most probably to DNA–protein interactions [86–89].

The biological importance of the Watson–Crick hydrogen bonding code has long been appreciated, however, DNA has a second code that can be read from the outside of a duplex without disruption of the base pairing interactions. The hydrogen bond donors and acceptors in the major and minor grooves of a B-form helix allow the base sequence to be deciphered by proteins that recognize specific DNA sequences. Sequence-specific DNA-binding proteins are essential in multicellular organisms where different parts of the genetic code must be accessed in cells with specific functions. Many of these proteins act as transcription factors, modulating either increased or decreased transcription of particular genes. In addition to the specific presentation of hydrogen bond donors and acceptors, the 5-methyl groups on thymine and 5mC are essential elements of the "second" DNA code [89].

In human cells, 2′-deoxyuridine-5′-monophosphate (dUMP) is enzymatically converted to thymidine-5′-monophosphate (TMP) using methylene tetrahydrofolate as the methyl donor. Folate deficiency or pharmacologic inhibition can increase the ratio of dUMP to TMP, and ultimately dUTP to thymidine-5′-triphosphate (TTP). DNA polymerases utilize dUTP as an alternative substrate for DNA synthesis, resulting in DNA containing uracil residues in place of thymine. Although the structure of DNA containing uracil in place of thymine is hardly discernable structurally from DNA containing thymine [90], multiple repair activities exist in higher organisms to remove uracil from DNA. As mentioned previously, uracil codes like thymine, therefore, the replacement of thymine by uracil would not be mutagenic. The abundance of uracil repair activities that appear to target misincorporated uracil residues, although they are not mutagenic and do not disrupt DNA structure, is perplexing until one considers the important role of the pyrimidine methyl group in sequence-specific DNA–protein interactions.

Experimental studies have directly examined the role of the thymine methyl group in sequence-specific DNA–protein interactions using electrophoretic gel mobility shift assays (EMSA). These studies have shown that the replacement of thymine by uracil can substantially reduce the affinity of the protein–nucleic acid interaction [87–89]. Replacement of the thymine with bromine, which is similar in size, restores the interaction [88], suggesting that the proteins have a hydrophobic pocket that accommodates the thymine methyl group. Similarly, oxidation of the thymine methyl group, forming HmU also disrupts sequence-specific protein interactions with DNA [89]. Most studies on DNA repair activities focus on their role in removing miscoding lesions that would otherwise promote miscoding and mutagenesis. The existence of an array of repair activities that act on apparently non-mutagenic lesions becomes more understandable when the important role of maintaining the "second" DNA code is considered.

4.5. The important role of the methyl group of 5-methylcytosine in DNA–protein interactions

Emerging evidence suggests that the specific placement of 5mC residues in human DNA is important for the control of gene transcription [91–96]. In contrast to thymine, the methyl group of 5mC is added to cytosine residues following DNA replication. The methylation is conducted by a DNA methyltransferase using *S*-adenosylmethionine (SAM) as the methyl donor. Methylation occurs predominantly in the CpG dinucleotide sequence. The process by which specific CpG dinucleotides are targeted for methylation is not understood completely. However, once the pattern of methylated CpG dinucleotides is generated, the corresponding methylation patterns are heritably transmitted to progeny cells because the maintenance methyltransferase recognizes the 5mC residue in the parental strand and methylates the opposing cytosine residue in the CpG dinucleotide on the progeny strand.

Earlier studies have revealed that the promoter regions of transcriptionally active genes tended to be less methylated than the corresponding regions of inactive genes. Furthermore, active genes appear to be in less condensed chromatin regions, whereas silent genes were found in highly condensed chromatin. The physical basis for these interactions is now becoming more clear. It is known that a class of DNA-binding proteins, the methyl-binding proteins (MBPs), have particular affinity for methylated DNA. It was recently demonstrated that methylation of cytosine residues at CpG dinucleotides increased the affinity for the MBPs by a factor of one hundred [96]. In addition to high affinity for methylated DNA, the MBPs also associate strongly with enzymes that covalently modify histone proteins. Together, the combination of histone protein modifications and DNA methylation are believed to modulate chromatin condensation and thus availability of specific genes for transcription. These modifications are collectively referred to an "epigenetic" modifications [97–99]. The biological importance of epigenetic mechanism is currently a topic of substantial investigation, as perturbations in the epigenetic code are associated with both the inappropriate silencing of tumor suppressor genes and the activation of transforming oncogenes in human tumors.

4.6. Halogenation of cytosine and perturbation of the epigenetic code

Halogenated pyrimidines have long been used as biophysical probes of nucleic acid structure. Recent studies, however, have indicated that halogenation of pyrimidines can occur in cells under physiological conditions [100–106]. In the human native immune system, neutrophils and eosinophils serve an essential role in defending against invasion by pathogenic organisms. Once activated, these cells generate powerful oxidizing species that can damage and destroy invading organisms. In neutrophils, myeloperoxidase converts hydrogen peroxide and chloride to hypochlorous acid (HOCl), whereas in eosinophils, eosinophil peroxidase generates HOBr. Both HOCl and HOBr can react with cytosine residues in DNA, forming 5-chlorocytosine (ClC) (Figure 7) and 5-bromocytosine (BrC), respectively. Both derivatives can be identified by their characteristic mass spectra.

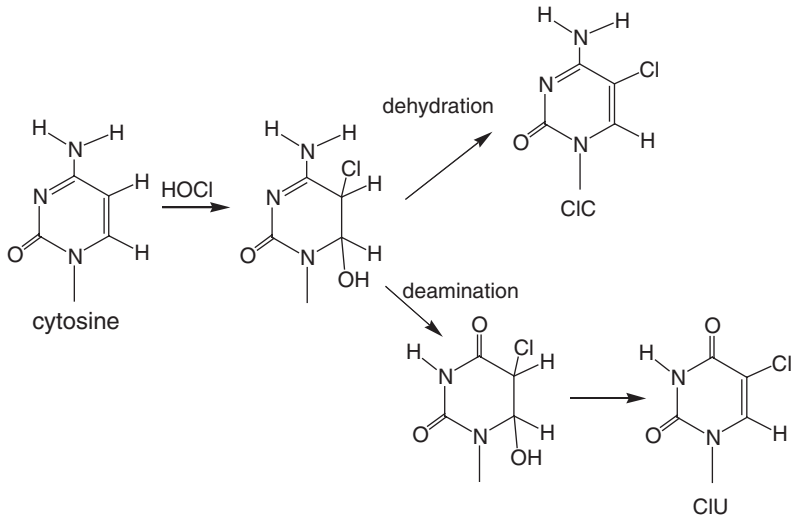

Figure 7 Products of reaction of cytosine with HOCl.

Recent studies have demonstrated that many forms of DNA damage, including the oxidation of 5mC to HmC or the oxidation of guanine to 8-oxoguanine, inhibit the binding of MBPs [96]. Interference with the binding of the MBPs could then prevent the recruitment of histone modifying enzymes and result in local chromatin decondensation and inappropriate activation of transforming genes.

In contrast very few forms of DNA damage would conceivably result in enhanced binding of MBPs leading to inactivation of tumor suppressor genes, as seen in virtually all human tumors. Up till now, the only cytosine methylation, histone modification and forms of DNA damage demonstrated to enhance MBP binding are the halocytosines, ClC and BrC [107]. Gel mobility shift assays have demonstrated that MBPs cannot distinguish oligonucleotides containing methylated cytosine from chlorinated or brominated cytosine. It is therefore possible that inadverted cytosine halogenation, collateral damage from attempts to protect from invading organisms, could induce a sequence of events that would result in the local condensation of chromatin and inadvertent silencing of tumor suppressor genes. It has also been demonstrated recently that the human maintenance methyltransferase, DNMT1, recognizes ClC and BrC as methylated cytosine residues [108,109]. Halocytosine-directed methylation of the progeny strand by DNMT1 could lead to heritable changes in epigenetic programming [108,109, 110,111,112].

5. Alkylation Damage of Pyrimidines

The pyrimidines may also be damaged by alkylation, even under normal physiological conditions. Numerous biological enzyme-mediated methylation

reactions depend upon SAM, including the formation of 5-methylcytosine in DNA and the methylation of specific residues on the histone proteins around which DNA is wrapped in the nuclei of eucaryotic cells. SAM is a sufficiently good methylating agent that it can inadvertently and non-enzymatically methylate DNA bases.

5.1. Alkylation of thymidine

As shown in Figure 8, thymine is most frequently alkylated in the O^4 position, although alkylation at the N^3 and O^2 positions has also been reported [113]. The O^4-alkylthymine analog has been used as a model of thymine when in the enol tautomeric form. However, the O-alkyl group can rotate between syn- and anti-positions relative to the N^3 position. The presence of the 5-methyl group of thymine would force the O-alkyl group predominantly syn- to N^3 [114–117]. Although O^4-methylthymidine is a model of the enol tautomer of thymidine, the presence of the methyl group in the hydrogen bonding face disrupts base pair

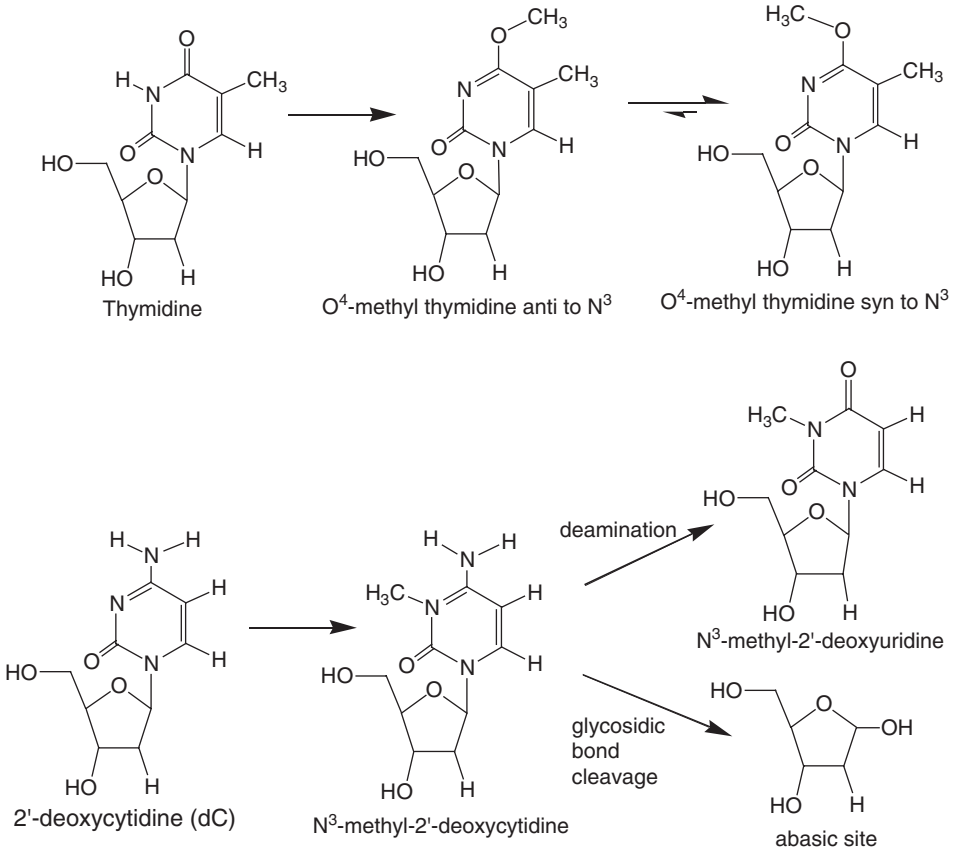

Figure 8 Alkylation of thymine and 2′-deoxycytidine and secondary hydrolysis of 3-methyl-2′-deoxycytidine.

formation. Methylation of thymine in the O^4 position does increase the frequency of mispair formation with guanine, however, the primary effect is to halt the progression of DNA polymerase [117,118].

5.2. Alkylation of 2′-deoxycytidine in the N^3 position

Alkylation of cytidine analogs occurs primarily in the N^3 position (Figure 9). The N^3 alkyl analog serves as a model of the imino tautomeric form of cytidine as mentioned previously. However, the p*K* of the N^3-alkylcytidine analogues is near 9 [35], so that the alkylated molecule is predominantly protonated at physiological pH. Indeed, N^3-methyl-2′-deoxycytidine has been used as a model to study the reactivity of protonated cytidine. Alkylation therefore drives protonation at physiological pH that then drives hydrolysis reactions, including glycosidic bond cleavage and deamination. The resulting N^3-alkyluridine derivatives are major blocks for the progression of DNA polymerase [117,118]. Methylation of cytosine residues can occur under normal physiological conditions. Alkylation of cytosine residues in the N^3 position can also occur by reaction with mutagens including acrylamide [119], (1-chloroethenyl)oxirane [120] and chemotherapy agents including phenyl acetic acid mustards [121].

5.3. Modifications to the N^4 amino group of 2′-deoxycytidine

The N^4 amino group of cytidine analogs can be directly alkylated (Figure 10). The N^4 amino group can also condense with activated aldehydes, including malondialdehyde [122] and tetrahydrofuran oxidation products [123,124]. The biological consequences of these adducts has not been fully revealed. The N^4 amino group can also be substituted indirectly. A variety of mutagenic amines, including hydroxylamine can displace the N^4-amino group of cytosine, resulting in the formation of the corresponding N^4-substituted analogs [125–127]. The

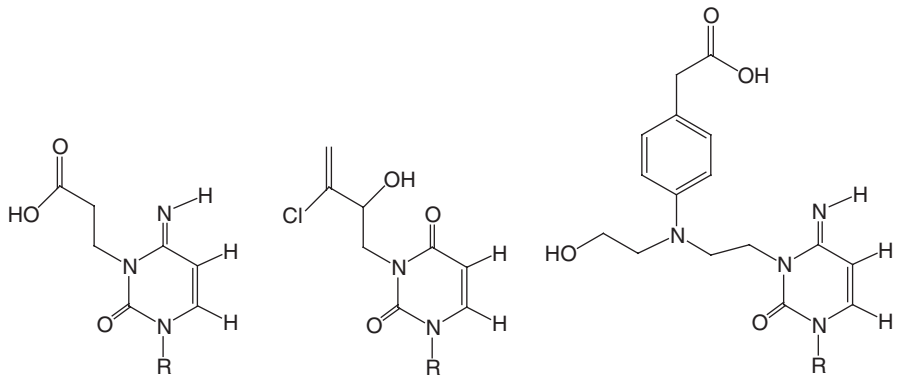

Figure 9 Reaction of the N^3 position of cytosine residues. Left: reaction product with acrylamide; Center: reaction product with (1-chloroethenyl)oxiraine; Right: reaction product with phenyl acetic acid mustard.

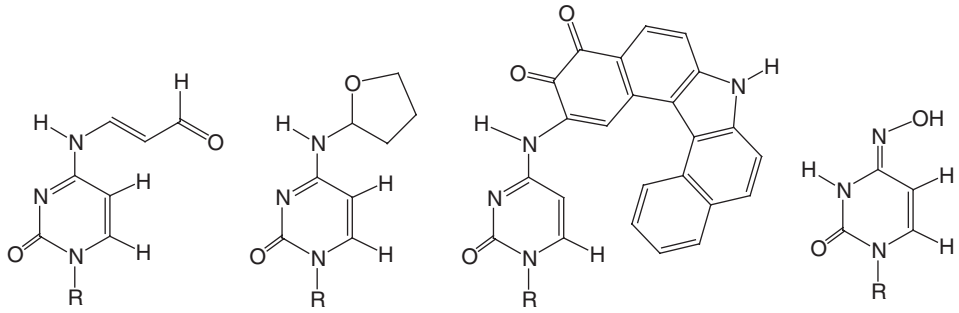

Figure 10 Reaction products with the N^4 amino group of cytidine analogs. From the left, reaction products of a cytosine residue with malondialdehyde, tetrahydrofuran, 7*H*-dibenzo [c,g] carbazole and N^4-hydroxylaminocytosine drawn in the imino tautomeric form.

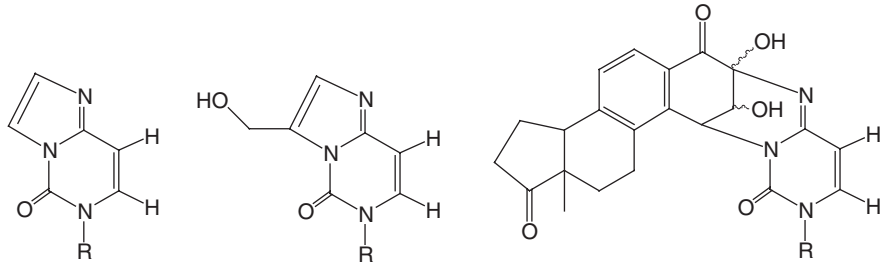

Figure 11 Reaction products with the N^3 and N^4 positions of cytidine analogs. From the left, reaction products of a cytosine residue with vinyl chloride, glycidaldehyde and equilenin.

replacement of a hydrogen atom with a heteroatom on the exocyclic amino group drives the base moiety toward the imino tautomeric form. Indeed, the 4-hydroxylamino analog of cytosine miscodes with adenine during polymerase-directed DNA replication. In NMR structural studies, the N^4-methoxy analog has been shown to form a base pair with guanine in a configuration approaching that of the Watson–Crick base pairs.

5.4. Alkylation of the N^3 and N^4 position of 2′-deoxycytidine

Some bifunctional agents are known to react with both the N^4 and N^3 positions of a cytosine residue, forming an adduct with an additional ring (Figure 11). Such agents including the environmental contaminants vinyl chloride, chloroethylene oxide, chloroacetaldehyde, glycidaldehyde, *trans*-1,4-dioxo-2-butene [128–130] forming etheno adducts. Adducts can also be formed with estrogen analogs including equilenin [131]. The biological properties of these adducts are not well-characterized, although the etheno adducts block DNA polymerase and promote transversion mutations [132].

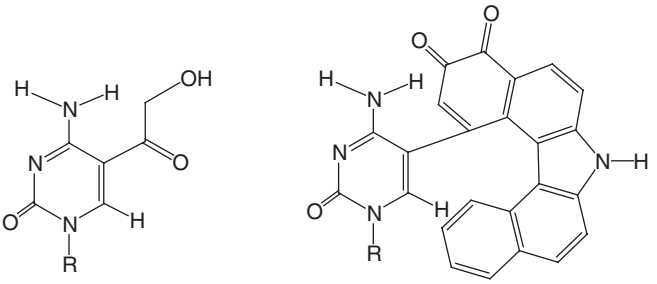

Figure 12 Reaction products with the C5 position of cytidine analogs. Left: reaction product with glyoxal. Right: reaction product with 7*H*-dibenzo[c,g]carbazole.

5.5. Alkylation of the 5-position of 2′-deoxycytidine

Less frequently, environmental chemicals can also react with the 5-position of cytidine analogs (Figure 12). These agents include 7*H*-dibenzo[c,g]carbazole that can react with cytidine analogs to form either N^4-amino substituted adducts or 5-substituted adducts [124] and glyoxal that can form 5-substituted cytidine or uridine analogs, the latter resulting from deamination of an intermediate [133]. The biological significance of the C5 adducts is as yet unknown. Although they would not probably cause miscoding and mutagenesis, they could interfere with the sequence-specific binding of regulatory proteins [134].

6. Repair of Damaged Pyrimidines in DNA

The repair of endogenous cytosine lesions in DNA proceed primarily by the BER pathway [135–138]. In the BER pathway, lesions are first identified and removed from DNA by a series of structure-specific glycosylases, followed by the removal of the damaged base. There are two groups of DNA glycosylases: DNA glycosylases that do not have apurinic/apyrimidic (AP) lyase activity are monofunctional glycosylases, and those with both glycosylase and lyase activity. In repair initiated by monofunctional glycosylases, the intermediate abasic site is acted upon by an endonuclease, APE1, which cleaves the sugar residue and generates 3′ and 5′ hydroxyl groups needed for repair synthesis and ligation. In contrast, the bifunctional glycosylases have an associated AP lyase activity that catalyzes either β-elimination or δ-elimination of the phosphate backbone. With both categories of glycosylase, the resulting gap is filled by DNA polymerase, usually DNA pol β in short patch BER, and the resulting nick ligated by DNA ligase III/XRCC1 heterodimer or DNA ligase I. Long patch BER is associated with the processive DNA polymerases, pol δ and ε, and other proteins including replication factor C and proliferating nuclear antigen. Long patch resynthesis generates several nucleotides by displacement of several downstream nucleotides. The resulting flap structure bearing is incised by flap endonuclease 1, and the resulting nick is sealed by DNA ligase I [173].

The first step in the process of repair is lesion recognition. The magnitude of this task is substantial in that the human genome experiences approximately 10,000 damage events per cell per day. However, the task is considerable complicated by the fact that these lesions are dispersed among the 10^9 bases of the human genome. The glycosylases must be very good at finding lesions, but further, the fidelity must be extremely high. If glycosylases could not distinguish damaged from normal bases with a fidelity exceeding 1 in 10^4, the glycosylases would create as much damage to the DNA as they would repair. Therefore, the mechanism by which glycosylase identify damaged bases is a subject of substantial interest.

The prototype for the pyrimidine glycosylase is uracil DNA glycosylase (UNG) [139–141]. Homologues of UNG are found in virtually all organisms. These glycosylases recognize predominantly, if not exclusively uracil residues in DNA. Uracil residues in DNA could be paired with adenine, guanine or be found in single-stranded DNA. UNG also recognizes a limited number of uracil analogs [146,165,179]. The selectivity for uracil over thymine results from the presence of a conserved phenylalanine residue that sterically blocks 5-substituted pyrimidines, allowing for discrimination against removal of thymine residues. Discrimination against cytosine removal is attributed to a conserved aspartate residue that forms hydrogen bonds with uracil, but clashes with the cytosine amino group. UNG is one of three glycosylases located on chromosome 12 in humans. The UNG gene can be spliced forming UNG1 or UNG2; UNG1 is targeted to the mitochondria, whereas UNG2 localizes in the nucleus [144,149], both of which have common catalytic domain, but different N-terminal sequences; UNG1 has 35 amino acids not present in UNG2 [147]. The 35 N-terminal amino acids are required for mitochondrial translocation [162].

A substantial number of studies have focused on characterization of DNA glycosylases [142–192]. Other glycosylases on chromosome 12 in humans are thymine DNA glycosylase (TDG) and single-strand-selective monofunctional uracil-DNA glycosylase (SMUG1). Interestingly, both are the same as UNG located in the long arm of chromosome 12. TDG in 12q22-24.1 and SMUG1 in position 12q13.1-14, respectively [148,162]. The function of TDG, or thymine DNA glycosylase, is proposed to be the repair of thymine residues that result from the deamination of 5mC. Thymine residues arising from 5mC deamination would be mispaired with guanine, and thus the context would provide the means of discriminating normal thymine paired with adenine from deaminated 5mC. Human TDG (hTDG) is homologous to the mispaired uracil glycosylase (MUG) found in *Escherichia coli*. MUG and hTDG are similar in both structure and function; both target mispaired uracil and thymine. The capacity to target mispaired thymine versus correctly paired thymine was originally attributed to the observation of specific hydrogen bonds between MUG and the guanine in the strand opposite to the targeted pyrimidine. Subsequent studies with a series of purine analogs failed to confirm the importance of specific hydrogen bonding interactions, but rather suggested that MUG was able to target mispaired thymine due to the reduced thermal stability of the thymine–guanine mispair. Studies with a thymine DNA glycosylase from the thermophile *Methanobacterium thermoautotropicum* are, however, consistent with selectivity based upon specific interactions with the "widowed" guanine [165].

MUG has a high preference for the excision of uracil over thymine. This effect was at first attributed to a steric effect as had been observed with UNG. However, subsequent studies with a series of pyrimidine analogs revealed that ClU, BrU and lU were very good MUG substrates, indicating that the substituent inductive effects, as opposed to substituent size dictated substrate preference [165]. Recent studies with hTDG indicate that substituent inductive properties strongly modulate substrate preference [186]. The rate of ClU cleavage is fifty times greater than that of the rate of uracil cleavage. The rate of BrU cleavage is very low, indicating that there is a size limit of the substituent interaction as well. This result may indicate that ClU, derived from HOCl-mediated chlorination of cytosine followed by deamination, is the primary physiologic substrate for hTDG.

The human SMUG1 targets a series of products derived from oxidation of the thymine methyl group, including HmU, FoU and CaU. The pyrimidine-binding pocket of SMUG1 is perplexing. SMUG1 does not cleave ClU or BrU, presumably due to size. However, the oxidized methyl groups are even larger, leading to the suggestion of a pocket containing a displaceable water molecule, displaceable by substituents that can be hydrogen bond, but not by halogens [179,183]. No glycosylase activity has been identified that recognizes and removes 5-chlorocytosine from DNA [182].

Human methyl-CpG-binding domain 4 (MBD4) is a thymine glycosylase that binds to T:G or U:G mismatch and does not have glycosylase activity on uracil paired with adenine, cytosine or thymine paired with adenine in double-stranded DNA substrates or uracil present in single-stranded DNA [160]. MBD4 has higher affinity for the CpG dinucleotide, especially those containing a 5mCpG:TpG mismatches, which is the deamination product of a 5mCpG dinucleotide [154]. Similar to TDG, MBD4 also removes 5-fluorouracil and weakly removes ethenocytosine [161]. It has been suggested that MBD4 and TDG act as "caretakers" of genomic fidelity at CpG sites [160]. The gene of MBD4 has been mapped to position 3q21.22 [161], the only one of four uracil-removing proteins identified to date that is not located to human chromosome 12.

Additional glycosylases have been identified as well that act upon damaged pyrimidines. These include NEIL1, NEIL2 and Fpg. These later glycosylases presumably also recognize damaged bases in unusual DNA structures including loop and hairpins. Further studies are required to understand the role of these glycosylases. NEIL1 and NTH1 are homologues of *E. coli* Endonuclease VIII (Nei) and Endonuclease III (Nth), respectively. Both are bifunctional DNA glycosylases; NEIL1 is associated with β and δ AP lyase activity, while NTH1 is associated with β AP lyase activity. Additionally, Endonuclease VIII is homologous to NEIL2 and NEIL3 [167,171,178]. NEIL2 is also a bifunctional glycosylase/AP lyase that catalyzes β and δ elimination reaction [163,167]. NEIL1 recognizes similar substrates as *E. coli* Nei, which excises adenine, guanine, formamidopyrimidines, and oxidized pyrimidines such as thymine glycol, 5-hydroxycytosine, 5-hydroxyuracil, 5,6-dihydroxyuracil, 5-formiluracil, 5-hydroxymethyluracil [163,167,193]. NEIL2 recognizes 5-hydroxyuracil, thymine glycol and alloxan, NEIL3 is able to excise thymine glycol [167].

Formamidopyrimidine DNA glycosylase (Fpg) is a DNA glycosylase with an associated AP lyase activity. NTH and NEIL proteins process a variety of pyrimidine-derived lesions, whereas Fpg protein acts primary at purine modifications. The Fpg glycosylese activity results in removal of 8-hydroxyguanine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, 4,6-diamino-5-formamidopyrimidine [156,184].

7. Conclusions

Pyrimidines are essential components of the nucleic acids of all organisms. Pyrimidines are subject to chemical damage by both endogeous agents and environmental toxins. Endogenous damage includes hydrolysis, alkylation and oxidation reactions, whereas exogenous agents can augment formation of endogenous products as well as cause the formation of chemically more complicated structures. Damage to pyrimidines in their many roles can result in loss of function for biomolecules, leading to cellular toxicity. Pyrimidine damage can also result in adducts that miscode during DNA replication, leading to genetic mutations. Emerging evidence also indicates that the structure of undamaged DNA is essential for modulating specific DNA–protein interactions needed for cell-specific control of gene expression. Specific modifications to DNA, through selective methylation of targeted cytosine residues, in concert with specific chemical modifications of histone proteins, are now recognized as essential components of the "epigenetic code". In addition to potential mutagenesis, some forms of DNA damage could have profound biological impact through interfering with or mimicking key aspects of the epigenetic code. Fortunately, most forms of DNA damage can be recognized and repaired through a complex array of glycosylases that recognize distinct features of damaged DNA. The consequences of different forms of DNA damage, as well as the complexities of the mechanism for damage recognition could soon be revealed at a molecular level.

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CHAPTER 7

Formation, Persistence and Significance of DNA Adduct Formation in Relation to Some Pollutants from a Broad Perspective

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Contents

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1. Introduction

Several epidemiological studies have demonstrated that exposure to chemical pollutants and food contaminants increase the risk of cancer. In general most of the substances become carcinogenic after conversion in the body by biotransforming enzyme into active metabolites. During the last 15 years, the aim of many studies has been to establish the potential relations which exist between biotransformation, repair and development of cancer when human beings are exposed to environmental pollutants. Relation between exposure of men or women to pollutants and birth defects or infertility has also been pointed out. In this chapter, the process involved in the biotransformation of chemicals into reactive metabolites and how these metabolites damage macromolecules, notably DNA, will be described. The significance of DNA adducts formation and persistence will also be discussed.

2. Steps of the Carcinogenic Process

Induction of tumour is the result of a cascade of events. It is a progressive process which could take several years (Figure 1).

2.1. Initiation step

The initiation of the carcinogenic process begins by damages to DNA. Different types of damage may occur: covalent binding of chemicals with DNA or modification of the DNA structure (break, crosslinks) (Figure 2).

Loss of base (1) arises often after alkylation (7) during repair; intra-strand cross-link (2) or inter-strand (6) are due to intercalating agents such as cisplatin, nitrogen mustard, chloroethylene; dimers of thymine are formed after exposure to UV (3); single-strand break (4) are induced by compounds such as bleomycin, adriamycin, some mycotoxins, X-ray and appear generally during repair; bulky DNA adduct (5) correspond to the covalent binding of the toxin or one of its metabolites on one base of the DNA. This initiation step is a quick process leading to irreversible change in the genetic material of the cells. When a xenobiotic enters the body, it will either be directly eliminated or biotransformed. Oxidation of chemicals by cytochrome P450 (CYP) or other enzymes such as prostaglandin-synthases, leads mainly to detoxification and favours the excretion of the substance. Nevertheless, in some cases, the oxidation induces the formation of reactive metabolites, which are nucleophilic and could react with the electrophilic sites of the macromolecules-forming adducts by covalent binding with deoxyribonucleotides (Figure 3). The formation of DNA adduct is not a biological lesion but a biochemical one. After two or three runs of cellular replication, a stable mutation could occur if the damages on DNA are not or badly repaired. DNA could be also damaged indirectly via reactive oxygen species (ROS) formed during biotransformation or lipoperoxidation. This leads to DNA breaks, DNA cross-link or DNA-protein

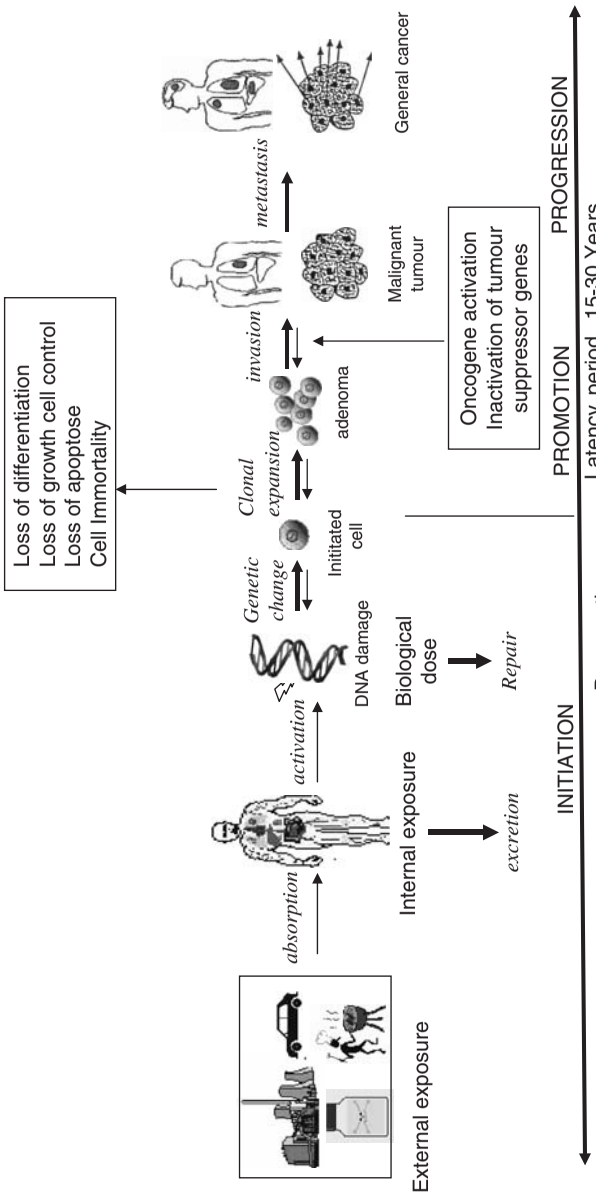


Figure 1 Tumoural process.

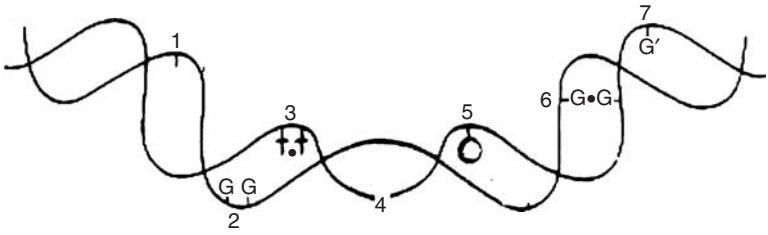


Figure 2 Examples of DNA damage.

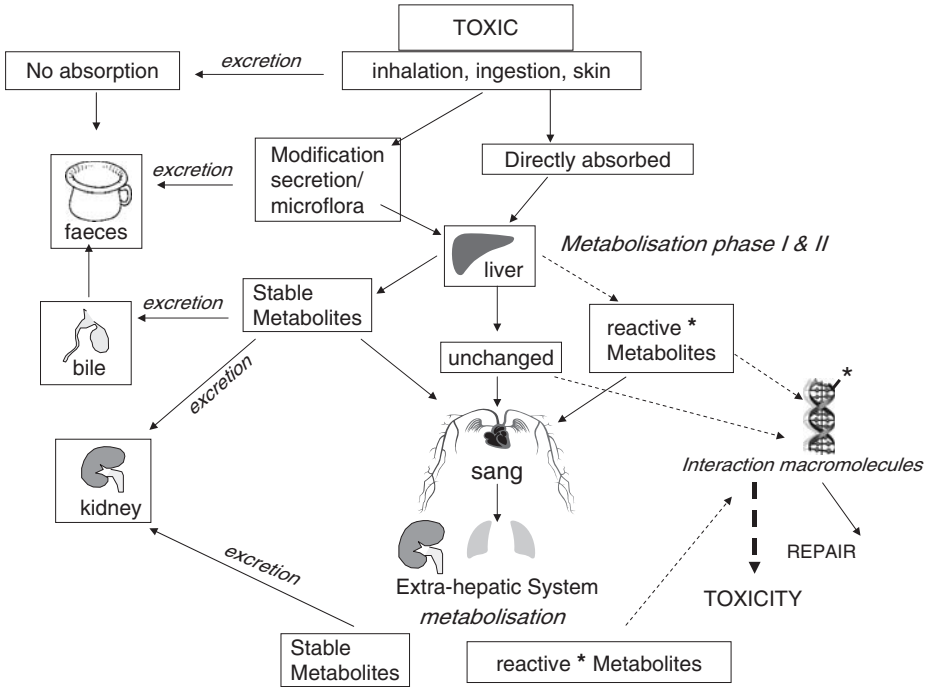


Figure 3 Distribution of xenobiotic and formation of reactive metabolites.

cross-link, exocyclic adduct corresponding to the formation of an addition cycle on one nucleotide (ethenobases or propanobase) and oxidated bases (8 hydroxy-guanine, 8 hydroxy-adenine, thymine glycol) [1].

2.2. Repair mechanisms

To maintain fidelity and integrity of genetic information, DNA damage should be repaired. Mammalian cells have several repair systems. Four different types of repair

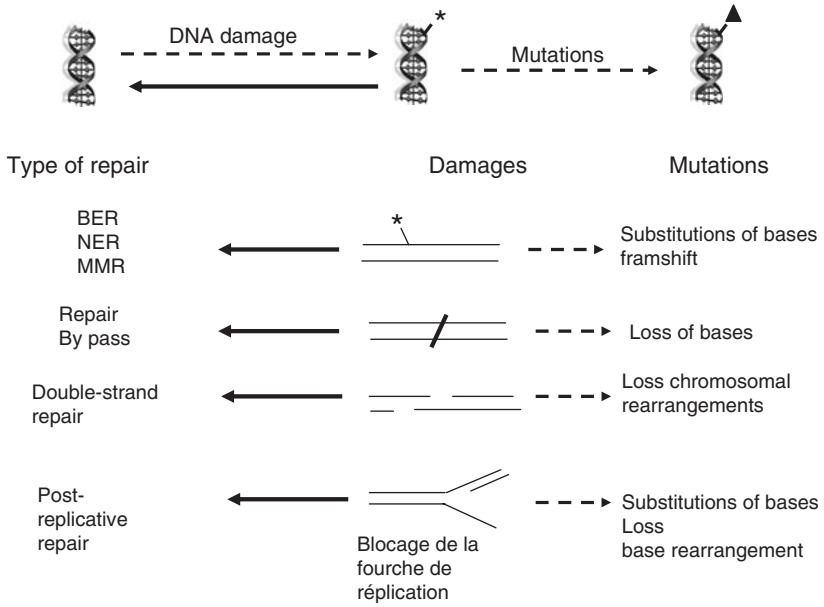


Figure 4 Mechanisms of DNA repair in human cells.

exist: base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR) and direct repair (for a review, [2]) (Figure 4).

2.2.1. BER

During BER, the damage base is eliminated by a glycosylase after excision by an endonuclease. The break generated is filled by DNA polymerase (β-pol), which synthesises a new strand using the opposite strand as template. There are several glycosylases (Table 1). Class I has only β-lyase activity, corresponding to cut DNA at the 3′ position. The 8-oxoguanine DNA glycosylase (OGG) excises oxidative damage. Alteration such as N7-aminoethyl guanine is unstable and is converted in open imidazole ring (2,6-diamino-4-hydroxy-5-*N*-methyl formamidopyrimidine, FaPy). This kind of lesion is eliminated by the FaPy DNA glycosylase (FPG). The glycosylases of class II delete the base, but do not cut the strand (3-methyl adenine glycosylase). Substrates such as thymine- and cytosine-glycols are eliminated by the endonuclease III (NTH human homologue). Two kinds of apurinic endonucleases (AP) exist. APE/ref-1 exhibits 2 activities: (i) repair by cutting the apurinic site in 5′ position then excision in 3′–5′; (ii) control of the redox properties of the transcription factor such as AP-1. APN1 corresponds to the endonuclease IV; it does not exhibit redox activity. APN1 cut the apurinic site in 5′ and has a 3′-phosphodiesterase activity. APN1 protects against damages induced by bleomycine and radiations. BER repairs oxidated, methylated and deaminated bases.

Table 1 Genes implicated in base excision repair (BER) and their properties

Genes	Functions
APE/ref-1	Cut apurinic site in 5′ position 3′–5′ exonuclease
APN1	Cut apurinic site in 5′ position 3′- phosphodiesterase
NTH	remove oxidated pyrimidines Lyase
FPG	remove open ring imidazoles (FaPy) remove 8-oxoguanine 3′–5′ β-lyase
OGG1	remove 8-oxoguanine remove open-ring imidazoles (FaPy)
XRCC	X-ray repair (insert directly accurate base)

2.2.2. NER

NER is the main mechanism involved in the repair of a large damage (dimer of thymine, bulky adducts such as aromatic amines or hydrocarbons and mispairing). It involves four steps:

- Recognition of the lesion (distortion of DNA),
- two helicases mark down the damaged area,
- incision on both sides by endonucleases and
- deletion of about 28 nucleotides followed by synthesis of a new strand by DNA polymerases δ and ε, then DNA ligase I ends the repair [3].

Some individuals having mutation of the genes coding repair enzymes have a higher risk to develop cancer [4]. Whereas BER takes in charge of only a few kind of frequent lesion such as oxidated bases, whereas NER plays a major role in repairing damages not corrected by BER (cross-link; exocyclic adducts, mispairing, bulky adducts). This repair (NER) also occurs when BER is saturated [1]. GG-NER repairs damages in non-transcribed region whereas TR-NER suppresses adducts in coding region.

The enzymes involved in NER are called XP because deficiency of repair induced xeroderma pigmentosum. There are 7 enzymes involved in NER: XPA, XPB, XPC, XPD, XPE, XPF and XPG. XPB or ERCC3 and XPD or ERCC2 are helicases allowing the opening of double strand and thus favouring endonucleases access. The enzymes XPG or ERCC5 (3′ endonuclease) and XPF or ERCC4 (5′ endonuclease) cut the lesions and remove about 30–50 base pairs. XPC is specific to GG-NER.

2.2.3. Mismatch repair

This system repairs mispairing arising from inaccurate replication or from recombination. Indeed, sometimes during replication a frame shift could unexpectedly

arrive notably when nucleotides are repeated. The enzymes involved are Mut S homologue-2 (MSH2) and Mut L homologue-1 (MLH1). Some colon cancers seem to be related to mutation of these genes [5].

2.2.4. Direct repair

O^6 alklyated guanines are eliminated by alkyl-guanine-DNA-methyltransferases. This enzyme transfers a methyl group only once. This prevents the formation of a cross-link with adjacent cytosine as could be observed with chloronitrosourea.

2.3. Promotion and progression

If a mutation induced by carcinogens, following bad repair, does not kill the cell when the cell and the DNA replicate, the mutation or the error in the genetic material will be transmitted to the daughter cell. These mutations or genetic recombinations lead to modification of normal expression either by activation of oncogenes, which convert normal cells into malignant phenotype or by inhibition of tumour–suppress, or by genes which stop or delay cell transformation (Figure 5). Tumoural transformation is the result of disequilibrium between factors stimulating cell division and factors inhibiting cell division. This disequilibrium is related to mutations of genes regulating cell growth.

Mutation could modify a factor favouring cell proliferation. In this case, cells will always be in division. More than 50 proto-oncogenes have been identified

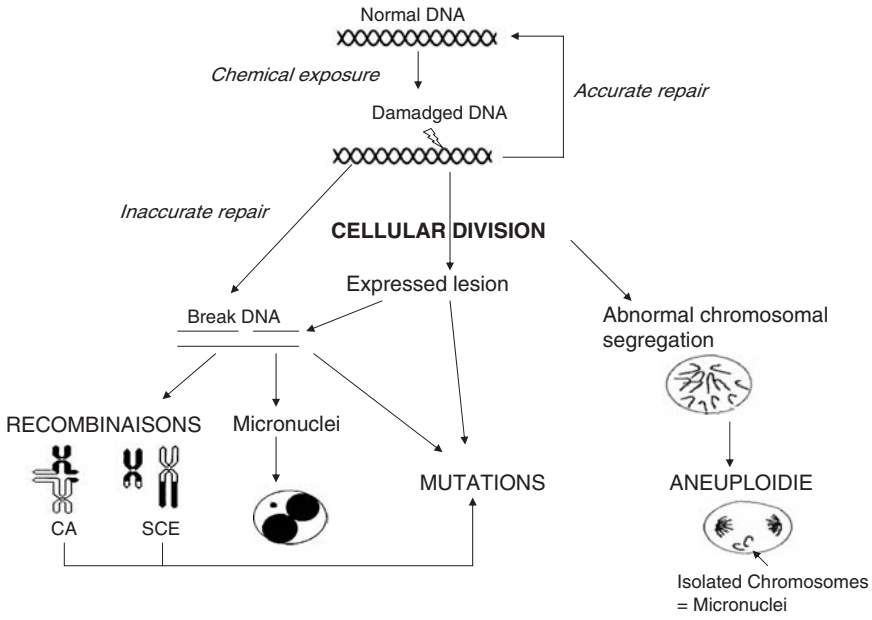


Figure 5 Biologic lesions due to bad repair.

(myc, ras, erb ….). In contrast, mutation could deactivate an inhibiting factor of cell division. For example, p53 protein stops cell cycle in G1phase giving cells time to repair damage. Non-repaired lesion at the beginning of the S-phase will be converted into mutations, responsible for cell transformation. When p53 is mutated, the cells lose one of its brakes and divide without stopping (Figure 6).

2.4. Biomarkers

Persistant DNA adduct lesions lead to several types of mutation which could be classified into two types:

- Macrolesions corresponding to change either in the number of chromosomes (aneuploïdie or polyploïdie), or in the structure of chromosome due to sister chromatides exchanges (SCEs).
- Microlesions corresponding to a modification in the nucleotides. These modifications are either qualitative (substitution of base pair) or quantitative (loss or addition of base) inducing a frameshift.

Biomarkers are useful tools allowing evaluation of aetiology and biological mechanism involved along the chain from exposure to pathology (Table 2) [6,7,8].

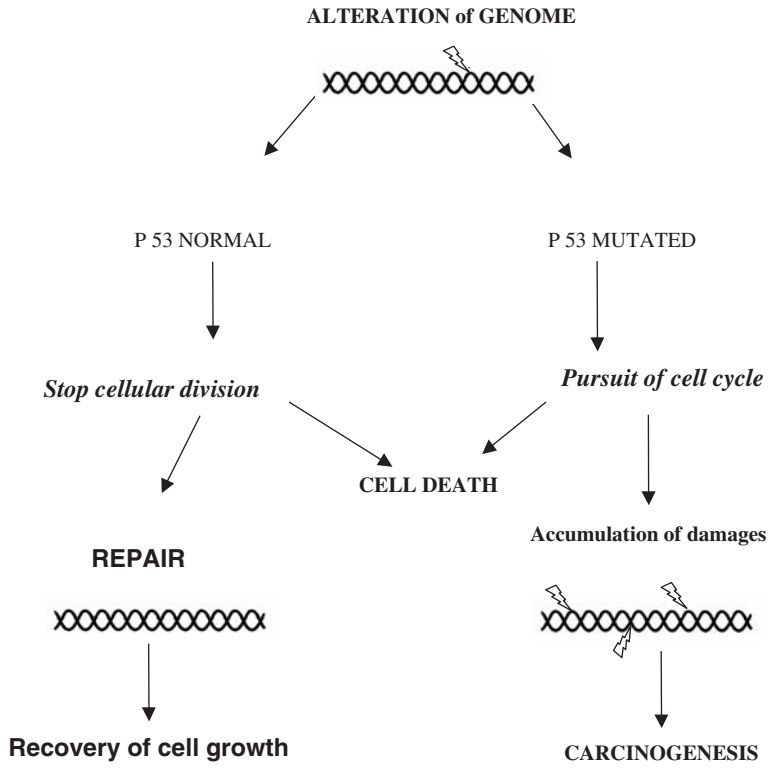


Figure 6 Role of p53 suppressor gene in carcinogenicity.

Table 2 Biomarker, temporal apparition and impact of enzymatic polymorphism

Dose/effect of the substance	Parameters	Temporal apparition	Impact of polymorphism
External dose	Concentration of the substance in environment (air, water, food, etc.)	Direct	No
Internal dose *(biomarker of exposure)*	Concentration of the substance in the biological fluids (blood, urine, etc.)	Minute-hour	Low
Biological effective dose	DNA adduct Protein adduct	Day-month	Yes
Biomarkers of susceptibility	Enzymatic induction (CYP, GST, etc.)	Minute-hour	Yes
Early biological effect *(biomarker of effects)*	Chromosomal aberration Micronuclei Aneuploïdie	Week-month	Yes
Biological response	Oncogene expression	Week/month	Yes
Late effect	Tumoural markers	Month/year	? (No study)
Disease	Tumour	Year	Yes

Measure of xenobiotic or one of its metabolites in biologic fluids gives notion of internal dose. Interaction between metabolites and macromolecules (DNA or protein) reflects biological effective dose. Analysis of biological lesion reflecting irreversible lesion corresponds to biological effect markers (chromosomal aberration; expression of gene). Evaluation of enzymatic induction is a biomarker of susceptibility. Altogether, biomarkers are useful to quantify the risk and help to understand inter-individual variation in the development of cancer.

Among many types of biomarkers, the most important aspect is the stability of the biomarkers with respect to time after exposure. Table 3 shows some examples of the stability of biomarkers of exposure for a persistent and non-persistent toxicity in case of acute exposure [9]. The situation is complicated more with chronic exposure because the compound could accumulate and constitute a mobile reserve of carcinogen.

The amount of alteration per genome is generally low and thus very sensitive methods are needed for detection. Detection of specific DNA damages is an essential tool for evaluating genotoxicity [10]. Persistence of these damages increases deleterious responses at all levels of the cell organisation. Detections of specific cell modifications also constitute biomarker of effect. Some tests are validated to allow the evaluation of DNA damage and repair capacity. Table 4 summarises cytogenetic biomarkers used to estimate exposure at workplace. The same biomarkers could also be used to follow environmental pollution or food contaminants. In the following section, relation between biotransformation and damages will be discussed.

Table 3 Fate of biomarkers of exposure in blood and urine

Biomarkers	Estimated detectable at post-exposure days (%)			
	Day 1	Day 10	Day 100	Day 1000
Exposure to persistent toxin				
Blood metabolites	77	64	50	13
Albumin adduct	151	84	18	4
Haemoglobin adduct	118	51	9	2
Lymphocyte DNA adduct	82	41	6	2
Exposure to non-persistent toxin				
Blood metabolites	4	0	0	0
Albumin adduct	170	80	15	0
Haemoglobin adduct	132	63	10	0
Lymphocyte DNA adduct	80	45	10	0

Table 4 Cytogenetic biomarkers used for the estimation of workplace exposure

Type of exposure	Compounds	Metabolites	Biomarker
Foundry Coke oven, Cheminey sweeper, Bus driver, car mechanic, Smokers	Polycyclic aromatic hydrocarbons (PAH)	Benzo(a)pyrene 7,8 diol, 9, 10 epoxyde (BPDE)	DNA adduct 8-hydroxyguanine Micronuclei (MN) Sister chromatides exchanges (SCE)
	Benzene	Hydroquinone Benzoquinone Benzene expoxide	DNA adduct Ethenobases Aneuploïdies Sister chromatides exchanges (SCE)
Smokers Dye industry	4-aminobiphenyl (ABP)	*N*-acetyl-aminobiphenyl	DNA adduct Haemoglobin adduct
Dye industry	Benzidine	*N*-acetyl-benzidine	DNA adduct *N*-(3′-phospho-desoxyguanosine-8-yl)-*N*′acetyl Haemoglobin adduct
PVC (polychlorid vinyl) factory	Vinyl chloride	Chloroethylenoxide Chloroacetaldehyde	Chromosomal aberation (CA) Sister chromatides Exchanges (SCE) Ethenobases p53 Mutation p21 Mutation
Rubber factory	1,3-butadiene	1,2-époxy-3-butene (MEB) 1,2:3,4-diepoxybutane (DEB) 3,4-epoxybutane-1,2 diol (EBD)	Haemoglobin adduct 2-hydroxy-3-butenylvaline Chromosomal aberation (CA) Sister chromatides exchanges (SCE) Micronuclei (MN) HPRT Mutation

3. Biotransformation and Genetic Alteration (DNA Adduct Formation)

3.1. Biotransformation pathways

The most lipophilic compounds are quickly absorbed via skin, lung and gastro intestinal tract. Some xenobiotics are excreted without any modification but often they are biotransformed in the organism into more polar compound which are easily excreted (via bile, urine, milk) while lipophilic compounds are stored in the tissues. Many enzymes biotransform the lipophilic compounds into polar ones. There are two kinds of reactions. (i) Phase I reactions are oxidations or hydrolysis leading to OH (phenol), SH (thiol), NH_2 (amine) or COOH (acid); (ii) Phase II reactions correspond to conjugation of the chemical or one of its metabolite with glucuronic acid, sulphate, glutathione, methyl and acetyl. After the biotransformation, the compounds are more polar and more water-soluble and thus are more easily eliminated. The biotransforming enzymes are mainly expressed in liver (10–50 times more than in the other tissues), even some of them are expressed in kidney, adrenals, testis, lung and placenta. They are located in the endoplasmic reticulum. The main pathway of biotransformation occurs in liver microsomes by the multi-enzymatic system of cytochrome P450 (CYP), but also by co-oxidation during prostaglandin synthesis. The enzymes involved are cyclooxygenases (COX) and lipooxygenases (LOX). These latter enzymes are extrahepatic. They are mainly present in kidney, lung and seminal vesicle. Several hundred compounds, structurally different but highly lipophilic are oxidated by the multi-enzymatic system following the global reaction:

$$NADPH + H^+ + O_2 + RH \longrightarrow NADP^+ + H_2O + ROH$$

Molecular oxygen and an electron provider are needed.

Several chemicals used in industries become carcinogenic after biotransformation. Several enzymes are polymorphic. This means that many alleles for one gene exist. The polymorphism corresponds to variation in the sequence of the gene, such as substitution, deletion, insertion and duplication. If modifications occur in coding region of the gene, the activity will be disrupted. Some alleles are responsible for modification of biotransformation activity. The number of allele for one gene ranges from 2 to 50. Table 5 summarises chemicals for which biotransformation involve polymorphic enzymes. The most important enzymes are CYP 1A1, 1A2, 2E1, NAT (*N*-acetyltransferase), GST theta (GSTT) or mu (GSTM), microsomal epoxyhydrolase (mEH), alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH).

Indeed some individuals develop cancer when they are exposed to a chemical product, whereas other workers in the same exposure conditions will not develop. The individual susceptibility is driven by metabolic profile and also by repair capacity (see below). Polymorphism corresponds to a variation of gene sequence such as nucleotide substitution, deletion, insertion or duplication. If this variation is in a coding region (exon), substitution of an amino acid can lead to an alteration of the properties of the protein. If this modification is in the promoter region, this will

Table 5 Chemical carcinogens used in industries and polymorphic enzymes

Chemicals	Enzymes
Polycyclic Aromatic Hydrocarbons (PAH): Benzo(a)pyrene (BaP)	*CYP 1A1, mEH, GSTM1, NQO1*
Aromatic compound: benzene, styrene	*CYP 2E1, GSTM1, NQO1*
Aromatic amine: aminobiphenyl (ABP), 2 naphtylamine, benzidine, hydrazine	*NAT1, NAT2*
Nitrosamines	*CY2E1, CYP2A6*
Alkanes, alkenes:	
Dichloromethane	*CY2E1*
Chloroethylene ethanol	*CYP 2E1, ADH, ALDH*
Ethylene oxide	*GSTT1*
1,3 Butadiene	*CYP2A6, CY2E1, mEH, GSTT1, GSTM1*
Formaldehyde	*ALDH, GST*

induce bad transcription. An incomplete or inactive protein could be synthesised. Table 6 summarises the main enzymes of biotransformation (and repair) which are polymorphic and the consequences. For example workers of banana plantation having the genotype CYPE1*2 develop more sister chromatid exchange, because over expression of CYP2E activity favour biotransformation into toxic compound [11].

3.2. Examples of environmental toxins

3.2.1. Benzo(a)pyrene

3.2.1.1. Main biotransformation pathways of B(a)P. Pyrolysis of compounds exposes individuals to PAH risk. First benzo(a)pyrene (B(a)P) is converted into an epoxyde (Figure 7) by addition of an oxygen atom on the double bound via the cytochromes P450, mainly CYP 1A1 and CYP 3A4. Three types of arene oxides are formed (4–5; 9–10; 7–8). These arenes could be hydrated and thus converted into dihydrodiol. This reaction is done by an epoxihydratase. In the meantime, arene oxide could be converted spontaneously to phenol. Several phenols (6; 2; 9 or 3 OH B(a)P) are formed directly by the action of CYP. In general, the phenols and diols are further conjugated to glucuronic acid or glutathione and eliminated. The 7,8 dihydrodiol B(a)P is converted into 9–10 epoxide-7,8 dihydrodiol during co-oxidation by prostaglandin synthase (PGHS). This latter metabolite (benzo(a)pyrene 9-10 epoxide-7,8 dihydrodiol, BPDE) is highly reactive against DNA and leads to the formation of DNA adduct on adenine (BPDE-10-N7dA) and on guanine (BPDE-10 N2dG). The phenols could be transformed into quinones by PGHS. These quinones can also generate DNA adducts. Two adducts are formed on guanine (B(a)P-6-C8dG; B(a)P-6-N7dG), and a third on the adenine (B(a)P-6-N7dA). GSTM1 deficiency increases formation of DNA, detectable in target tissue, and also in lymphocytes and buccal cells.

Table 6 Characteristic of polymorphism of enzymes (biotransformation or repair) and associated biological activity

Enzyme	Allele (halotype)	Mutation	Enzymatic activity
CYP1A1	*1	Wild type	Normal
	*2 or Msp1	T>C 6235	Increase
	*3 or Ile/Val	A>G4889	Increase
	*4	T>C 5639	Increase
	*5	C>A 4887	?
CYP1A2	*1	Wild type	Normal
	*2	C>A 2964	Inducible
CYP2D6	*1	Wild type	Normal (EM)
	*3 or A	Base deletion	Null
	*4 or B	Mutation of base	Null
	*5 or D	Base deletion	Null
	*6 or E	Base deletion	Null
	*15 or T	Insertion of base	Null
CYP2E1	*1 or C1	Wild type	Normal
	*3 or C2	C>T 1019	Increase
GSTM1	*1 or active	Wild type	Normal
	*0	deletion	null
GSTT1	*1 or active	Wild type	Normal
	*0	deletion	Null
GSTP1	A	Wild type	Normal
	B	Iso104 Val	Decrease
	C	Iso104 Val; Val 113Ala	Decrease
NAT2	*4	Wild type	Fast
	*5a	T341C; C481T	Slow
	*5B	T341C; C481T, A803G	Slow
	*5C	T341C; A803G	Slow
	*6A	G590A	Slow
	*7B	C282T; G857A	Slow
EH	R or *1	Wild type	Normal
	H	Exon 3-Tyr113HIS	Decrease
	Y	Exon 4-His139Arg	Decrease
ALDH2	*1	Wild type	Normal
	*2	Glu487 Lys	Decrease
NQO1	*1	Wild type	Normal
	*2	C609T exon 6	Null
ERCC1		A >C 8092	Decrease
ERCC2 or XPD		199 Ile > Met	Decrease
		312 Asp > Asn	Decrease
		751 Lys > Gln	Decrease
hOGG1		326 Ser > Cys	Decrease
XRCC1	TGGG	194 Arg > Trp	Decrease
	CAGG	280 Arg > His	Decrease
	CGAG	399 Arg > Gln	Decrease
XRCC3		241 Thr > Met	Decrease

Notes: CYP, cytochrome P450; NAT, *N*-acetyltransferase; GST, glutathione transferase; EH, epoxyhydrolase; ALDH, aldehyde-dehydrogenase; NQO NAD(P)H quinone oxydoreductase; ERCC1, excision repair cross-complementing; hOGG, human 8-OH-guanine glycosylase; XRCC, X-ray repair cross-complementing.

Figure 7 Main route of B(a)P biotransformation and formation of DNA adduct.

Biotransformation of B(A)P involves several polymorphic enzymes, notably CYP 1A. Liver expresses the highest CYP activities even though other tissues also contain some CYP. Families 1, 2 and 3 play the main role in the biotransformation of xenobiotics and thus in the formation of reactive metabolites leading to DNA adduct formation. Some of them are polymorphic.

3.2.1.2. Polymorphism of CYP1A1. The CYP1 family includes three members: 1A1, 1A2 and 1B1. Four alleles have been identified for the CYP1A1 family: CYP 1A1*1 is wild type; CYP1A1*2 corresponds to replacement of thymine (T) by cytosine (C) at 6235 position of the intron 7 and leads the formation of a new site of restriction for Msp1; CYP1A1*3 or Ile/Val corresponds to replacement of adenine (A) by guanine (G) at position 4889 of exon 7; CY1A1*4 corresponds to replacement of T by C at the 5639 position of the intron 7.

Two polymorphisms of CYP1A1 are linked to the difference in the aryl hydrocarbon activity (AHH). A positive correlation exists between AHH activity in human lung and the formation of DNA adduct [12,13]. Substitution of C by A in exon 7 induces change of aminoacids (Ile/Val) in the region of heme fixation. Val mutant have an activity two times higher than wild type. The polymorphism corresponding to transition of T to C downstream of polyadenyl site is called *MspI* allele (Table 7).

Significantly more benzopyrene-diolepoxyde (BPDE)-DNA adducts are formed in lung and blood cells of individuals with homozygosis for the *MspI mutation* (*2A/*2A) compared to heterozygotes (*1/*2A ou *1/*2B) as well in smokers [14] as in foundry workers [15]. In the same way, smokers with Val polymorphism have a high amount of DNA adduct [16]. Chimney sweepers with *MspI* homozygosis have more DNA adduct and micronuclei in lymphocytes T [17]. More PAH-DNA adducts are observed in newborns with the genotype CYP1A1, MspI [18,19,20] or in individuals exposed to PAH via atmospheric pollution [21,22]. In a study on Italian coke oven, the highest amount of PAH-DNA adducts were observed in the workers having the genotype CYP 1A1 Ile/Val, whereas the genotype MspI does not modulate the DNA adducts [23]. The amount of 1-OH pyrene excreted by was higher for MspI policeman [24].

3.2.1.3. Polymorphism of glutathione-S-transferase (GST)

3.2.1.3.1. GSTM1. Glutathione mu (GSTM1), highly present in liver, is known to detoxify arene oxide such as BPDE. Foundry workers exposed to B(a)P in air (5 ng/m^3) have a high level of DNA adduct, but the polymorphism of GSTM1 seems not to play a role in this study [15]. Pavanello et al. [25], in contrast, demonstrated that coke oven workers deficient in GSTM1 have significantly more DNA adduct in lymphocytes than the other workers. The difference is more pronounced when they are exposed to high level of PAH [23]. This correlation between DNA adducts and GSTM1 null was also observed in chimney sweepers [25] and bus drivers in Copenhagen [26], CA are also higher in these bus drivers [27]. GSTM null bus driver has also higher HPRT mutation [28]. DNA adduct increased with the concentration of PAH in air only when the coke oven workers were GSTM1 null [14,35].

Table 7 Relation between CYP 1A1 polymorphism, DNA adduct and exposure to PAH

Type of mutations	Exposure	Individuals	Tissues	Biomarker	Relation	Publication
Exon 7	Smoker		White blood cell	DNA adduct	+	[16,29]
Ile/Val Val/Val	Coke oven	25	Lymphocytes	DNA adduct	+	[30]
Ile/Val	Coke oven	65 exposed	White blood cell	DNA adduct	+	[31]
Ile/val	Coke oven	76 exposed 18 control	Total blood	DNA adduct	+	[23]
Ile/Val	Foundry	100	Total blood	DNA adduct	–	[15]
Ile/Val	Environmental pollution, passive smoker	194 student	Lymphocyte	DNA adduct CA*	+ +	[21]
MspI	Smoker		Blood	BPDE-DNA adduct	+	[14]
MspI	Coke oven	89 exposed 44 control	Blood	BPDE-DNA adduct	+	[32,33]
MspI	Chimney sweep	69 exposed 18 control	Lymphocytes	DNA adduct	+	[17]
MspI	PAH	Newborn	Lymphocyte	DNA adduct	+ ++ when GSTP1 Ile/Ile	[22]
Ile/Val	Smoker, passive smoker	Newborn (199)	Placenta	DNA adduct	+/–	[34]

Notes: MspI, CYP1A1*2; Ile/Ile or Ile/Val, CYP1A1*3; exon 7, CYP 1A1*4; CA, chromosal aberration.

3.2.1.3.2. GSTP1. Significantly more micronuclei were observed in lymphocytes of coke oven workers who are GSTM1 null, having a high EH activity. This could be explained by the fact that the main detoxifying pathway of PAH is through glutathione conjugation via GSTM1 [36]. GSTP1 is also involved in the detoxification of BPDE. GSTP1 is highly expressed in epithelial cells of several tissues, notably lung and oesophagus. Substitution of isoleucine with valine in exon changes the activity of the enzyme. Patients with lung cancer having the 104 Val allele present much more DNA adduct in the non-tumoural part of the lung than the patient with homozygote for non-mutated gene [37]. Recently, analysis of 141 coke oven workers in comparison to 66 non-exposed workers showed a highest frequency of micronuclei (MN) in coke oven workers having the genotype GSTP1 Val 104/Val 104 compared to those having Ile 104/Ile 104 or Ile 104/Val 104 [36]. This confirms the data showing a significantly higher amount of BPDE-DNA adduct in coke oven workers having GSTP1 Val 104 [38].

3.2.1.4. Combination of several polymorphism. Smokers having a combination of the genotype CYP1A1 (MspI) and the genotype GSTM1 null develop lung cancer more frequently, and the occurrence of p53 mutation is higher [39,40]. Significantly, more BPDE-DNA adducts in their lungs were detected [12,41]. Same conclusion could be drawn for coke oven workers [42]. Indeed, eighty-nine coke oven workers (35 smokers, 33 ex-smokers, 18 non-smokers) have been recruited and categorised in function of exposure to PAH in air. BPDE-DNA adducts were observed in 52% of samples. Only for 5/74 individuals who are GSTM1*0/*0, no DNA adduct were observed. In contrast, 69/74 individuals who are GSTM1*0/*0 have a large amount of DNA adducts. The quantity of DNA adduct is related to CYP1A1 genotype. The highest amount of DNA adduct was observed in the workers who are GSTM1 null and CYP1A1 (*1/*2 or *2/*2), the lowest was observed in the individuals who are GSTM1 null and CYP1A1 (*1/*1). No DNA adducts are formed in workers with active GSTM1 and CYP1A1 (*1/*2). Chimney sweepers having the CYP1A1 (*MspI*) have more BPDE-DNA adduct when they are deficient in GSTM1. For these individuals, a correlation between DNA adducts and micronuclei could be established [17]. Level of DNA adducts due to urban pollution was higher in individuals having a combination of the genotypes CYP1A1*2A and GSTM1 null [21]. The same is true for individuals having the combination of CYP1A1*2A and GSTP1 null or CYP1A1*2A and EH lent (His139His). Analysis of DNA adducts in lymphocytes of 160 mothers and their newborns indicated significantly more DNA adduct in newborns (heterozygote or homozygote) for the allele CYP1A1*2, or for newborns who have GSTP1 Ile/Val or Ile/Ile. The amount of DNA adduct is four times higher if the young ones have a combination of the two genotypes GSTP1 Ile/Ile and CYP1A1*2 [22].

3.2.1.5. Polymorphism of repair system. Coke oven workers having a low repair capacity (XPC PAT +/+ and XPA-A23A) develop more BPDE-DNA adducts compared to workers who did not have these genotypes [43]. In addition this is amplified when the workers are GSTM1 null. The genotype XPD-Asn312Asn is

also associated with increasing BPDE-DNA adduct in smokers [44] and policemen exposed to PAH [45]. Coke oven workers with the wild-type halotype CGGG of XRCC1 are protected against chromosomal aberration. In contrast, the risk is increased in workers having the halotypes TGGG or CGAG [46].

3.2.2. Benzene

In petroleum industry (refinery, transport and distribution) or rubber factory, workers are exposed to benzene. The first step, as previously explained, is the formation of an epoxide followed by formation of quinone able to react with DNA (Figure 8). Individuals having a high CYP 2E1 activity and a low NADPH quinone reductase (NQO) have a high risk of hematotoxicity, directly correlated to the formation of quinone derivatives [47,48]. Higher SCEs are formed in worker exposed to benzene when they are GSTT1 null [49].

Measurements of benzene, muconic acid, catechol and hydroquinone *S*-phenyl mercapturic acid in urine are biomarkers of internal dose. Benzene could also be measured in blood and exhaled air.

3.2.3. Vinyl chloride and acrylamide

Formation of epoxide in the aliphatic chain is again the first step in biotransformation of vinyl chloride (VMC) (Figure 9), acrylamide (Figure 10) or acrylonitril and also butadiene. Exposure to vinyl chloride occurs during the processing of items in PVC (polychlorovinyl). The reactive metabolite is the chloroepoxyethane leading to the formation of several DNA adducts (and protein adduct). This formation is under the control of CYP 2 E1, ALDH and GSTT1 [50]. The first step is the oxidation by CYP2E1 of vinyl chloride in chloroethylene oxide (CEO) and chloroacetaldehyde (CAA), both responsible for ethenobases [51]. CAA is a substrate of ALDH2. Workers exposed to VMC and deficient in ALDH2 (homozygotes or heterozygotes) have a higher frequency of SCE than workers with normal ALDH [52]. Deficiency in ALDH activity is due to change of amino acid in position 487 (Glu > Lys). VMC is hepatotoxic and induces human cancer. GSTT1 protect individuals against toxicity [53]. Vinyl chloride forms ethenobases leading to mutation of the gene TP53. This mutation is linked to over-expression of the protein p53 [54]. A correlation between expression of this protein and exposure to vinyl chloride has been observed. Nevertheless, different over-expression after same exposure to vinyl chloride between individuals is explained by the genetic polymorphism of XRCC1 enzyme repair [55]. Thus, heterozygotes Arg–Gln or homozygotes Gln–Gln are more at risk than homozygote Arg–Arg. The odd ratio (OR) is 1.73 (95% IC 0.93–3.22) and 3.95 (95% IC 1.68–9.28), respectively. In addition the difference is even more pronounced if the workers are CYP2E1 c2 [56].

Glycidamide (epoxide of acrylamide) also induces the formation of two adenine adducts, two guanine adducts and one cytosine adduct (Figure 10).

1,3 butadiene is a contaminant of petrochemical industries and could be found in air via car exhaust emission. This substance is biotransformed by CYP (2E1, 2A6) into 1,2-epoxy-3-butene (EBO) than in 1,2:3,4 diepoxybutane (DEB) [57].

Figure 8 Biotransformation of benzene.

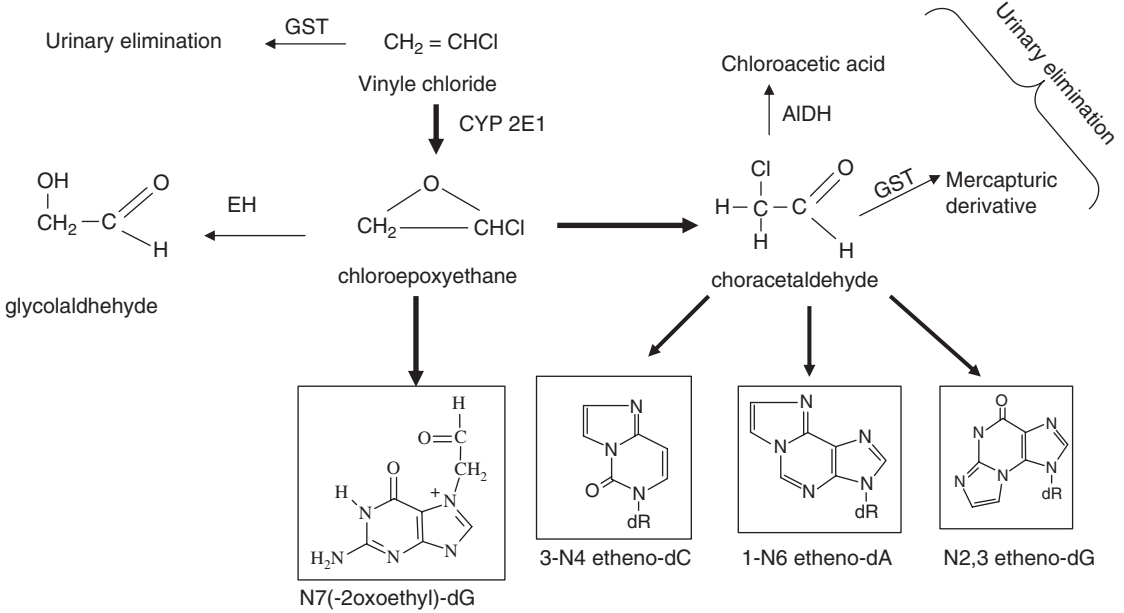


Figure 9 Biotransformation of vinyl chloride and formation of DNA adduct.

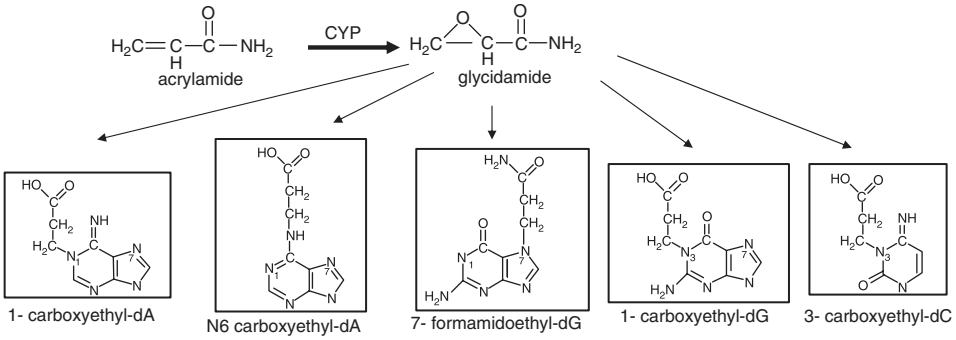


Figure 10 DNA adduct formation during biotransformation of acrylamide.

DEB will be converted by epoxyhydrolase into 3,4-epoxy-1,2 butanediol (EBD). The latter induces SCEs and micronuclei in lymphocytes. Human sensitivity to 1,3 butadiene was related to EH polymorphism [58,59]. Sensitivity to DEB has allowed Wiencke et al. [60] to classify the individuals as sensitive or resistant to SCE induced by DEB. All workers sensitive to DEB are GSTT null, whereas workers having wild-type GSTT are resistant [61]. A consequence of this damage is an increase of cell proliferation [62]. DEB also induces formation of micronuclei in workers sensitive to DEB [61]. A correlation between occurrence of SCE and 1,2-epoxy-3-butene was observed in lymphocytes of workers lacking GSTT [63,64]. In the same way, workers lacking GSTM1 exposed to dimethylformamide or epichlorohydrin during process of items in PVC have more SCEs [65].

3.2.4. Ethylene oxide and styrene oxide

Ethylene oxide is the simplest epoxide, which is an intermediate product of chemical synthesis of ethylene glycol. This compound is also used in hospitals for disinfection. Ethylene oxide is a substrate of GSTT1 and GSTM1, allowing its urinary excretion. Ethylene oxide is electrophilic and binds nitrogen in position 7 of guanine (*N*-7-(2-hydroxyethyl) dG) as well as valine and histidine. DNA adduct formation is modulated by GST polymorphism [66]. Ethylene oxide is carcinogenic for human beings.

Biotransformation of styrene (Figure 11) leads to the formation of an epoxide on the aliphatic chain via CYP 2B6, 2 E1 and 1A2. Epoxyhydrolase hydrolyses the epoxide into styrene glycol and is transformed after that by alcohol deshydrogenase (ADH) into mandelic aldehyde and then into mandelic acid by aldehyde deshydrogenase (ALDH). An additional oxidation induces the formation of phenylglyoxylic acid. These two latter metabolites could be found in urine [67]. Styrene oxide and ethylene oxide could be bound to proteins mainly to histidine of haemoglobin and albumin [68]. They also bind DNA on the 7 position of guanine. Glutathione conjugation is a minor pathway for the biotransformation of styrene. Biotransformation of styrene involves several polymorphic enzymes including CYP 2E1, GSTP1 and epoxyhydrolase [69].

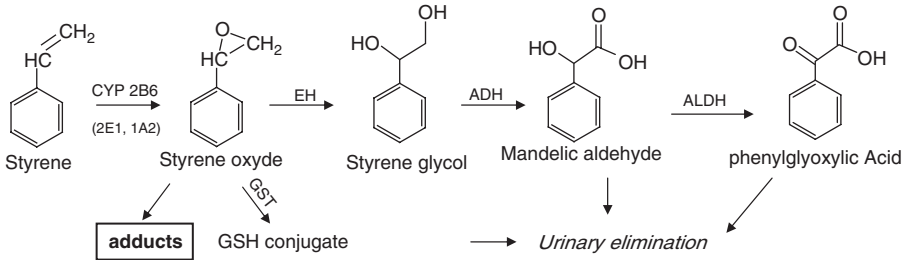


Figure 11 Biotransformation of styrene.

7,8 oxide styrene (SO) and monoepoxide butene (MEB) are substrates of GSTM1 and induce formation of SCE. Much more SCE are found in workers who are GSTM1 deficient [70,71,72,73]. Occurence of SCE induced by SO is higher in worker GSTT1 null [74]. EH plays also a role in the formation of MN [75,76]. In an epidemiological study conducted on Chinese workers exposed to butadiene, more haemoglobin (Hb)-adduct (*N*-(2,3,4-trihydroxybutyl) valine) appeared [77]. This level is higher in workers who are GSTM1 null or GSTT1 null [78]. In the same way, the amount of *N*-1-(2,3,4-trihydroxybutyl) adenine (*N*-1-THB-Ade) is significantly higher in workers exposed to butadiene who are GSTM1 deficient. The level of adduct is proportionate to the exposure [79]. The polymorphism of the glutathione theta (GSTT) modulates the toxicity of halogenated alkane and ethylene oxide which is decreased, whereas the toxicity of vinyl chloride is increased [81,82,83], related to exposure to styrene, in resin factories which induces DNA adduct. Occurence of MN induced by exposure to styrene is higher in lymphocytes of workers GSTT1 null [84]. Interestingly, workers with GSTP1 wild-type homozygote (A/A) and EH 1/1 (slow EH) are protected from this damage [85]. Exposure to styrene is also revealed by measurement of haemoglobin valine correlated with DNA adduct and micronuclei. All these three parameters measured in 44 workers exposed to styrene in comparison to 44 non-exposed workers pinpointed the modulation due to polymorphism. The frequency of micronuclei in mono-nucleated lymphocytes (MNMC) is higher for individuals with XRCC3 Met 231 allele. Workers with the allele XRCC1 Gln 399 have higher amount of both the types of micronuclei (mono-nucleated or binucleated) [86].

3.2.5. Dimethyl sulphate and formaldehyde

Dimethyl sulphate (DMS) is mainly used as methylating agent in organic synthesis. It is hydrolysed into mono-methyle and methanol and further biotransformed into formaldehyde (Figure 12). DMS directly methylated the DNA on N7 or O6 of the guanine and N3 of the adenine. Formaldehyde (or formol) is highly reactive. It is used in producing resin and glue, but it is also used in hospitals for disinfection due to its anti-microbial activity. It makes protein and DNA cross-links [87]. Formaldehyde is detoxified by glutathione conjugation and by the action of aldehyde dehydrogenase [88].

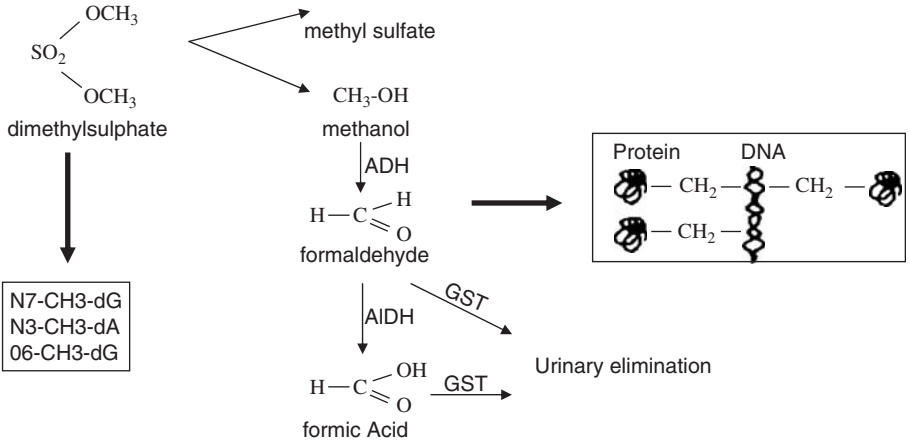


Figure 12 Biotransformation of dimethyl sulphate and formaldehyde (formol).

3.2.6. Aminobiphenyl and benzidine

The main sources of aromatic amines are the industries of dye, rubber, textile, leather and paper; from the production of polyurethane, epoxy-resin; chemical industries; hairdressers; photography industry; and exposure to tobacco. For the majority of aromatic amines, metabolic activation requires the formation of *N*-hydroxyarylamines and *N*-hydroxyarylamides. This involves enzymatic *N*-oxidation reactions, which, depending on the aromatic amine substrate are catalysed preferentially by CYP 1A2 and CYP 3A4, or by flavin-containing mono-oxygenases. Most of these hydroxylated derivatives undergo further metabolic activation to form highly reactive *N*-*O*-esters. The metabolic pathways of 2-naphtylamine, 4-aminobiphenyl (Figure 13) and benzidine are similar and involves CYP 1A2 and NAT1, leading to the formation of highly reactive nitrenium that makes DNA adduct responsible for bladder cancer [89]. Individuals fast acetylators are at higher risk, notably if their urine is acidic [90].

Acetylation is an important route of metabolism for aromatic amines and there are a wide variety of substrates. Acetylation is notable in that the product may be less water-soluble than the parent compound and thus the acetylated metabolite being less soluble in urine, crystallised out in kidney tubules. These enzymes which catalyse the acetylation reaction (NAT) are highly expressed in cytosol of several tissues. These acetylations are responsible for the activation of aromatic amines into reactive intermediates such as *N*-acetoxy-acetylamine giving rise to nitrenium or carbonium ions able to cross-react with C8 of the guanine [91]. It has been found that the acetylation of certain compounds in human beings shows wide inter-individual variation due to genetic basis. The repartition is bimodal with slow and fast acetylator. Two genes exist: NAT1 and NAT2. Occurrence of slow acetylator is higher in patients suffering from bladder cancer; in contrast, the proportion of fast acetylator is higher in patients suffering from colon cancer [92]. Activity of NAT1 is correlated with aromatic amine-DNA adduct in bladder [93,94,95], and in

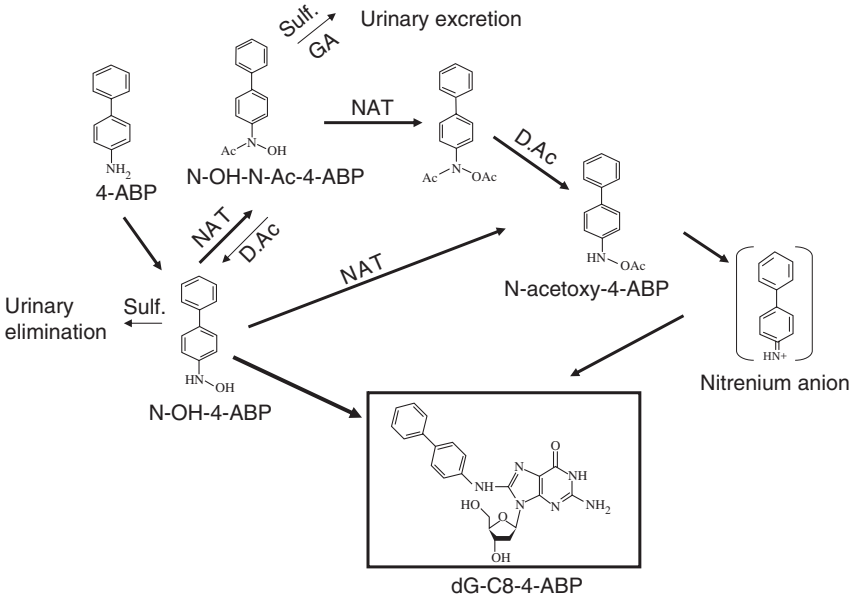


Figure 13 Biotransformation of 4-aminobiphenyl (ABP) and formation of guanine adduct.

pancreas [96]. When patients are fast acetylators via NAT1 and slow acetylators in liver via NAT2, the amount of DNA adduct in exfoliated bladder cells are much higher [94]. This is due to the fact that individuals who are fast acetylators via NAT2 detoxify better amine aromatics in liver, and thus the hydroxylated derivatives which will be converted into reactive metabolites by NAT1 do not reach epithelial bladder cells. The amount of aminobiphenyl (ABP)-Hb adduct is dependant of the activity of NAT2. Slow acetylators have more ABP-Hb [97,98]. In the same way, workers in a dye factory exposed to aromatic amines have significantly more ABP-Hb adducts when they are slow acetylators (NAT2) [99]. CYP1A2 is involved in *N*-hydroxylation of aromatic amines and thus is a risk factor for the development of bladder cancer because it favours the formation of ABP reacting with Hb. The individuals who combined the characteristic fast CYP1A2 and low acetylation capacity (slow NAT2) exhibit much more Hb adduct [100,101].

3.3. Examples of some food contaminants

3.3.1. Ochratoxin A and pentachlorophenol

Man-made chlorophenols (CPs) are widely distributed in the environment due to their agricultural and industrial uses as insecticides, herbicides, fungicides and wood preservatives. In the presence of CYP450, pentachlorophenol (PCP) undergoes oxidative dechlorination to form tetrachlorobenzoquinone (TCBQ) that reacts with deoxyguanosine (dG) [102] and other DNA bases [103] to form benzethenotype adducts. Activation by enzymes with peroxidase activities furnishes the

phenoxyl radical that can covalently attach to the C8-site of dG to generate the C8-OPCP adduct [104,105]. In the presence of glutathione (GSH), redox cycling of the phenoxyl radical with thiyl radical generation will yield a GSH disulphide anion radical that can in turn reductively activate O_2 to generate the superoxide radical anion ($O^{2\cdot-}$) that can generate free ferrous ion (Fe^{2+}). In the presence of H_2O_2, the Fenton reaction will yield hydroxyl radical and cause oxidative DNA damage; a pathway regarded as a contributor to peroxidase-driven toxic effects of phenolic xenobiotics [106]. Free Fe^{2+} may also act directly on PCP to cause a reductive dehalogenation process and produce the carbon-centered radical that reacts with dG to yield the C8-CPCP adduct (Figure 14). Thus, bioactivation of PCP by peroxidase enzymes yields radical intermediates (phenoxyl radicals and quinone) that cause oxidative DNA damage and covalent DNA adducts through attachment to the C8-site of dG. Electrophilic benzoquinone (TCBQ) intermediates are produced by CYP450 activation, and TCBQ is known to form covalent adducts with DNA, which may contribute to PCP-mediated carcinogenesis [107]. In the same way, ochratoxin A (OTA), which is a natural chlorophenol is also biotransformed into quinone-OTA, able to react with DNA and other hydroxylated derivatives (for recent review, see [108]). Comparisons of DNA adduct in reactions with OTA or OTHQ alone versus microsomes indicate that

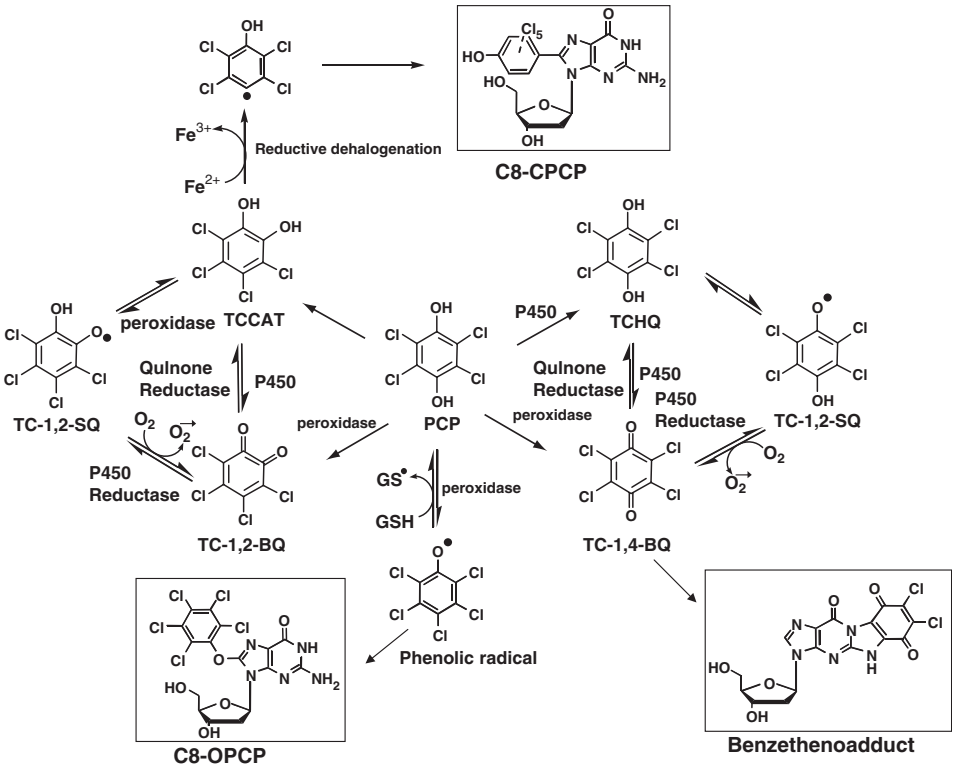


Figure 14 Biotransformation pentachlorophenol (PCP).

quinone metabolite generates DNA adduct which could be important for carcinogenicity [109]. The DNA adduct pattern is dependent on the expression of biotransforming enzymes notably those enzymes implicated in the metabolism of arachidonic acids such as cyclooxygenase (COX), lipooxygenase (LOX) and epoxygenase, which is related to CYP450 2C11, differently expressed in male and female (for recent review, see [110]). The carcinogenicity of OTA is mainly due to CYP 2C [111]. Induction of CYP2C9 favours formation of OTHQ-related adducts, whereas LOX and COX2 induction favours the formation of the dG-OTA adduct [109,112]. These results are completely consistent with the intermediacy of the OTQ electrophile in activation of OTA by CYP450, which yields OTHQ-related adducts, and radical intermediates from peroxidase activation that generates dG-OTA [112]. Interestingly, CYP 2C is induced by sex hormones [113] and regulated by CYP 3A4 [114] abundantly expressed in the human liver, intestine and kidney. The CYP 3A5*1 allele was more prevalent in patients suffering from Balkan endemic nephropathy (BEN) for which OTA is a plausible aetiologic agent, with a frequency of 9.38% compared to 5.36% in controls and was associated with a higher risk for BEN (OR 2.41) [115]. Several data also pointed the implication of glutathione in OTA genotoxicity [116,117]. Andonova et al. [118] have shown that carriers of at least one GSTM1 wild-type allele (positive conjugators) were more prevalent among BEN patients compared to controls (OR 7.92) (Figure 15).

3.3.2. Aflatoxin B1

Aflatoxin B1 is the main secondary metabolite formed by *Aspergillus flavus* and *parasiticus* in several crops and nuts. This compound is responsible for liver cancer. AFB1 is activated to AFB1 8, 9-exo epoxide, which binds predominantly to the N7 position of guanine, resulting in apurinic site, or stable imidazole ring-open AFB1-N7-guanine (AFB1-Fapy). These adducts excreted in urine are good marker of initial hepatic DNA adduct levels and are related to critical mutation. In humans the exo epoxide is formed via CYP 3A4, whereas AFM1 is formed by the action of CYP 1A2. Exo epoxide AFB1 is detoxified by GSTM1. Thus, polymorphism of this enzyme could explain the difference of susceptibility of some individuals (for a review, see Pfohl-Leszkowicz, 1999 [119]; Castegnaro & Pfohl-Leszkowicz, 1999 [120]) (Figure 16). XRCC1 is involved in the repair of AFB1-DNA adduct [121].

3.3.3. Aristolochic acid

Aristolochic acids (AA) are derivatives of 3,4-methylenedioxy-10-nitro-1-phenanthrenecarboxylic acid. They are found in the roots and leaves of *Aristolochia* species. They have been used since antiquity in obstetrics and for their anti-inflammatory properties. Until 1981, AAs were marketed as pharmaceutical specialities consisting of plant extracts from species such as *A. Clematitis* or in form of mixture of AAI and AAII. Following the discovery of AA as a strong forestomach carcinogen when administered orally to rodents [122,123,124,125], these drugs have been withdrawn from the market in most countries, but not in Asia. AAs have been used to investigate stomach carcinogenesis in rodents as a model for human cancer.

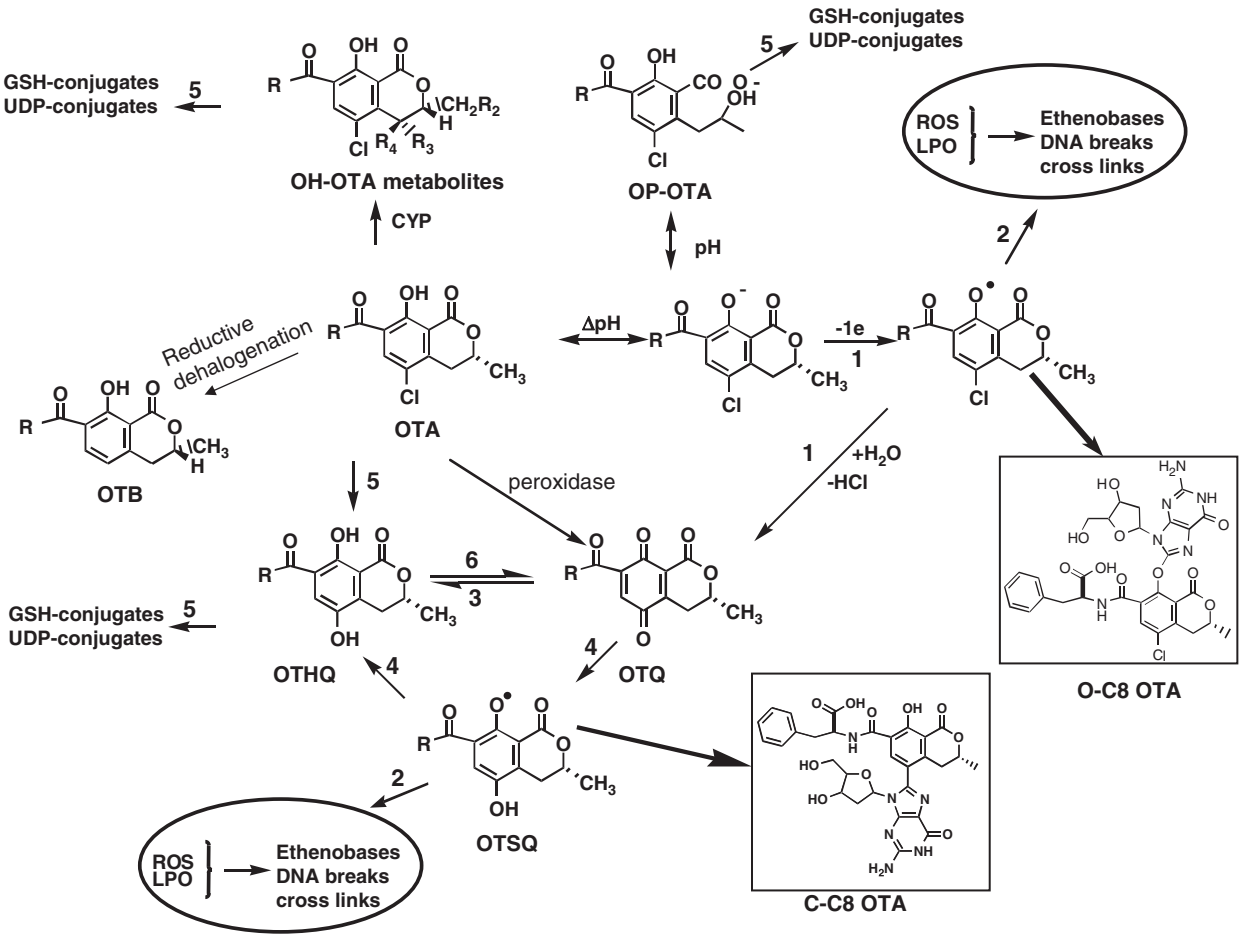


Figure 15 Biotransformation of OTA.

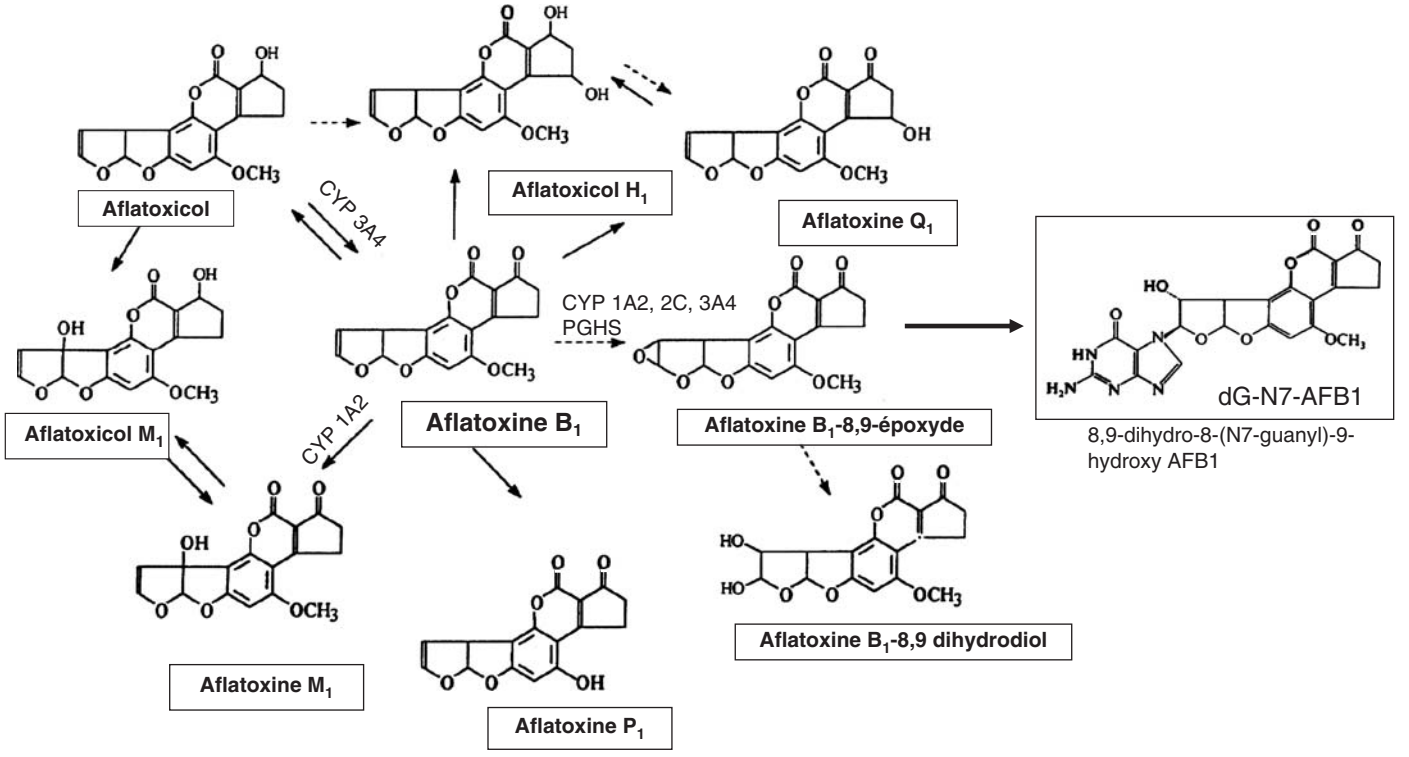
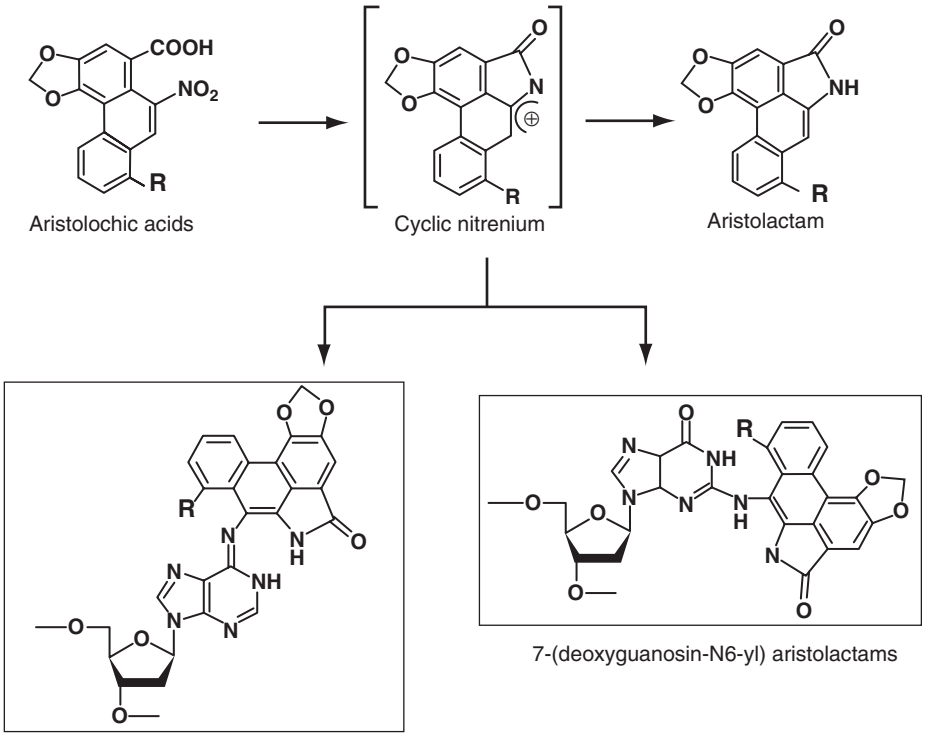


Figure 16 Biotransformation of aflatoxin B1 and formation of dG-N7-AFB.



R = OCH_3, aristolochic acid I (aristolactam I) ; R = H, aristolochic acid II (aristolactam II); R = OH aristolochic acid Ia (aristolactam Ia)

Figure 17 Biotransformation of aristolochic acids (AAs).

The organ specificity coupled with the short latency period in the carcinogenesis of AA are advantages of this *in vivo* model [126,127]. The two main compounds (AAI and AAII) differ only by the presence of a methoxy group in AAI.

The hydroxylamine derivatives are metabolically formed either by partial reduction of the nitro group or by oxidation of amino function (via CYP 1A). Both mechanisms resulting in the formation of a cyclic *N*-acylnitrenium ion which is converted into aristolactam or cross-react with DNA giving guanine (7-deoxyguanosine-N2-yl-aristolactam) and adenine adduct (7-deoxyadenosin-N6-yl-aristolactam) (Figure 17). AA induces mutation at the first adenine of codon 61 of H-ras in all forestomach and ear duct tumours of rats treated with AAI or plant extract.

3.3.4. Heterocyclic amine food mutagens

Heterocyclic aromatic amines (HAAs), arylazides and 4-nitroquinoline 1-oxide are mutagens. Because of their wide spread occurrence in cooked meat products, HAA (Figure 18) may contribute to common forms of human cancers including

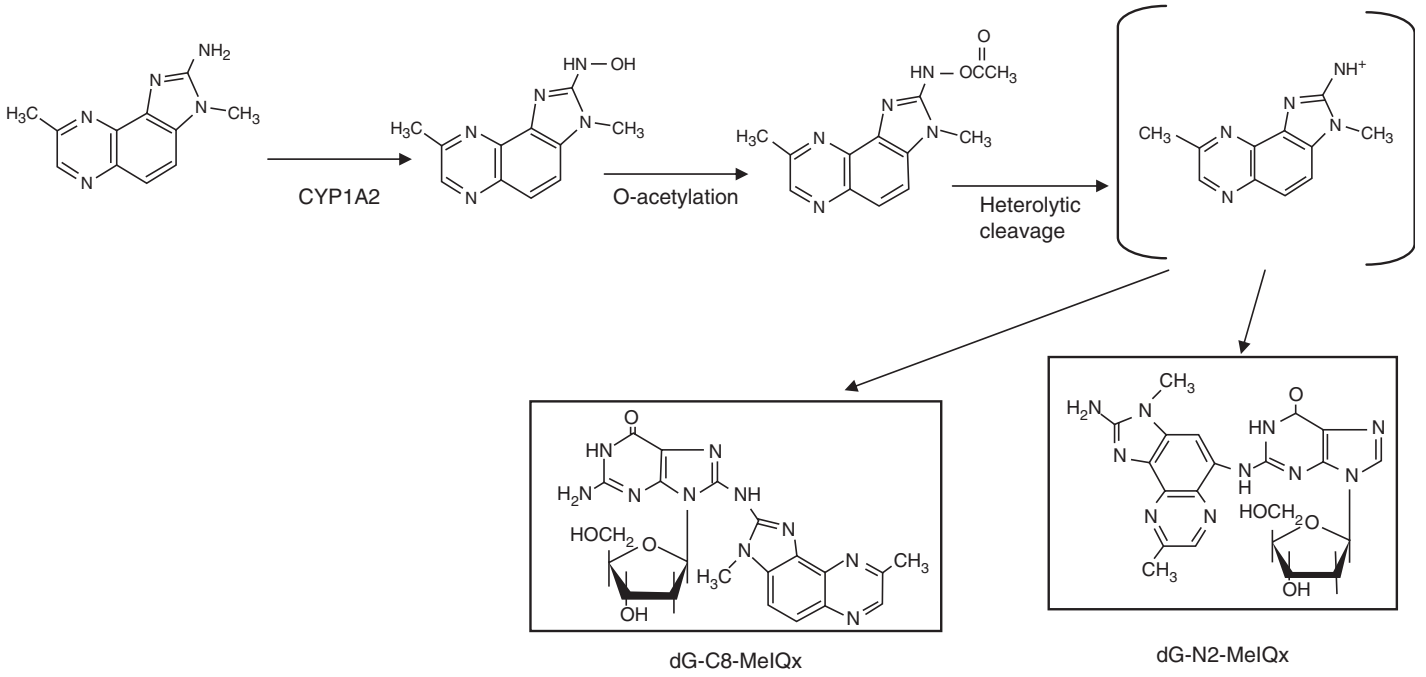


Figure 18 Biotransformation of 2-amino-3,8-dimethylimidazolo[4,5-f]quinoxaline (MeIQx).

colorectal, prostate and breast which are associated with frequent consumption of diets high in meats and fats. Their genotoxic properties are attributed to a common intermediate, the arylnitrenium ion which is the ultimate species that reacts with DNA adduct in the same way as aminobiphenyl does (see above). The first step of biotransformation of amine aromatic is the hydroxylation of the amines mainly via CYP 1A2 in liver and to a lesser extent by CYP1A1 and 1B1 in extrahepatic tissues, while cytosolic diaphorase catalyses the reduction of 4-nitroquinoline 1-oxide to *N*-hydroxy metabolite [128]. DNA binding of the *N*-hydroxy compounds is greatly enhanced by the generation of reactive esters, such as the *N*-acetoxy derivates, which undergo heterolytic cleavage to produce the reactive nitrenium ion–acetate anion pair (Figure 19). NAT, sulphotransferase, phosphorylase and l-propyltRNA synthase contribute to the bioactivation of *N*-hydroxy-HAA through formation of reactive esters. The *N*-acetoxy derivatives of IQ and MeIQx are highly unstable with lifetimes of seconds, whereas *N*-acetoxy-PhiP is relatively more stable and has been characterised by mass spectrometry. These metabolites formed covalent binding with guanine via C8 [129]. In addition to dG-C8 adducts, adducts are formed at N2 atom of guanine at the C5 position of IQ and MeIQx (Figure 18, [129]). For most HAAs, DNA adduct formation is greater in liver, which may be attributed to the high levels of CYP1A2 expression. In contrast to other HAAs, the amount of adducts formed with PhiP are relatively low in liver compared to extrahepatic tissues; adducts are particularly elevated in colon, pancreas, prostate

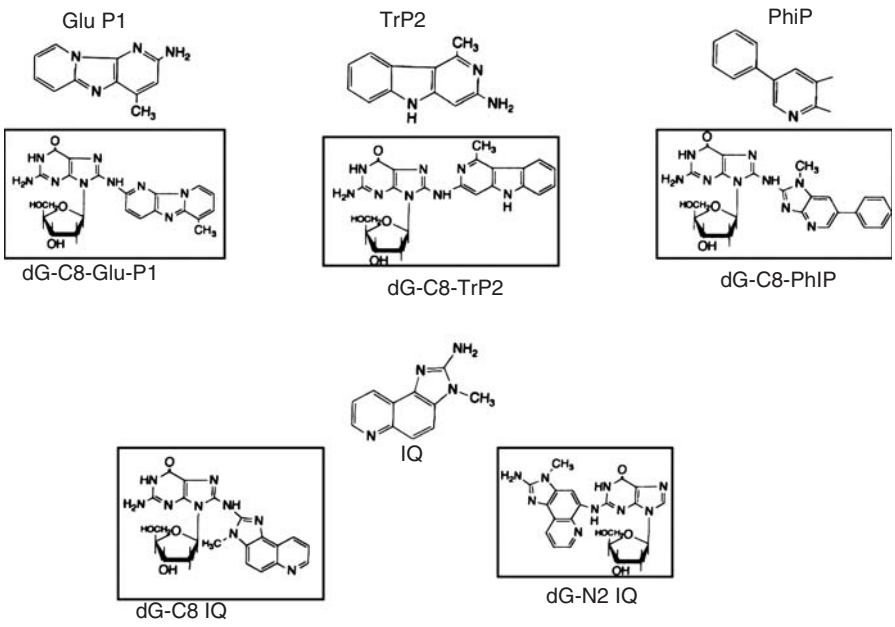


Figure 19 Structure of several heterocyclic aromatic amines and their corresponding DNA-adducts: Glu-P1, 2-amino-6-methyldipyrido[1,2-a:3′,2′-d]imidazole; TrP2, 3-amino-1-methyl-5H-pyrido[4,3-b]indole;PhiP, 2-amino-1methyl-6-phenylimidazo[4,5-b]pyridine; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline.

and the mammary gland of female rodents. GST responsible of detoxification of reactive PhiP has been shown to be high in liver. This may explain the relatively lower quantity of PhiP-DNA adduct in this tissue.

3.4. Impact of genetic polymorphism on DNA adduct formation and subsequent toxic response

Individual susceptibility to several pathologies following exposure to chemical or environmental pollutants is indisputably linked to the capacity of individual to biotransform xenobiotics and to repair initial damages. Genetic polymorphism of several enzymes (biotransformation, repair) affects the level of expression, the structure or the catalytic activity of the enzymes. The different studies demonstrated clearly that the factors of susceptibility (biotransformation, repair) reflect the individual response to exposure to genotoxic agents.

3.4.1. Scheme of interaction between several polymorphisms

Competition between several biotransformation pathways is a crucial factor of the initial DNA damage of carcinogen (formation of DNA adduct; chromosomal aberration and subsequently tumour development). Amount of reactive metabolites reacting with DNA, result from the equilibrium between activating pathways and detoxifying pathways. In the same way mutations result from the equilibrium between DNA adducts formation and repair systems (Figure 20, [130,131,132]).

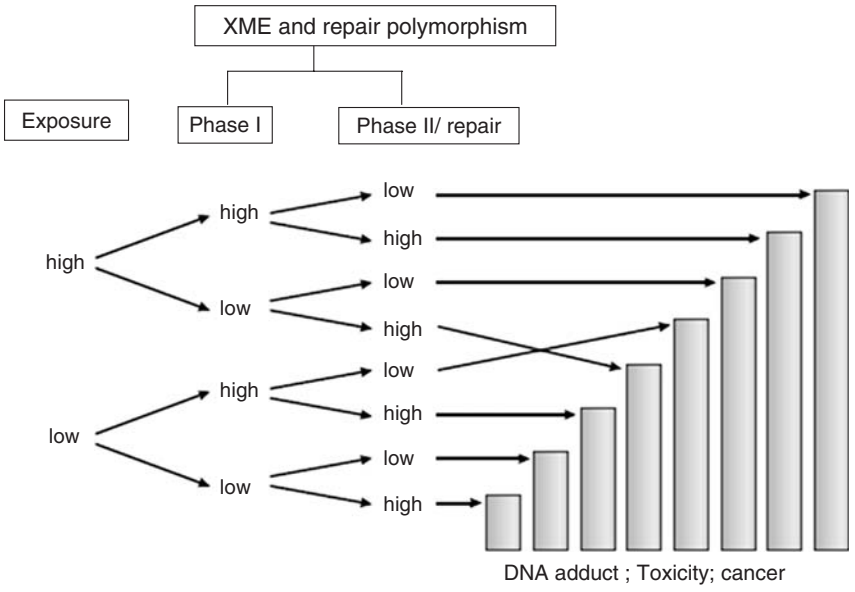


Figure 20 Scheme of classical interactions between exposure and biotransformation via phase I enzymes; phase II enzyme and repair enzymes, and cancer risk.

3.4.1.1. Interaction between phase I enzyme and phase II enzymes. On the whole an individual who has large amount of biotransforming enzyme from phase I (CYP, COX …) but has low activity of enzymes from phase II (glutathione conjugation; glucuronidation …) will form more reactive metabolites which in turn lead to more DNA adducts. In contrast, low activity of phase I enzymes combined with high conjugation enzyme protect individuals against damage [32,33].

Moreover, synergies between several polymorphism and carcinogen risk exist [21,41,42,133]. Intensity and time of exposure also play an important role on expression of polymorphism, because some enzymes are inducible [134].

3.4.1.1.1. CYP1A and GST. Biotransformation of pollutant plays a main role in the carcinogenic process and also in the teratogenic process, notably because the detoxifying system is immature in foetus and newborns. For example newborns lack GSTM1 [18,19,20]. It has been observed that newborns who are heterozygote for CYP1A2 (MspI), and who have been exposed during pregnancy to carcinogen have significantly more chromosomal aberration [135].

Thus, individuals exposed to PAH via atmospheric pollution or smoking have significantly more DNA adducts when they are GSTM1 null. They have also more chromosomal aberrations (as well micronuclei as SCEs) [136]. Similar results are observed for workers exposed to butadiene or styrene. Low NAT2 individuals have more ABP-DNA adducts in their bladder than other workers with fast NAT activity. Although fast NAT individuals are protected against bladder cancer, they are more susceptible to larynx, lung or colon cancer. In general, combination of several polymorphisms such as CYP 1A1 and GSTM null; or slow NAT and GSTM1, increases not only lung cancer risk but also biomarkers of effects (DNA adduct, micronuclei and SCEs) related to PAH exposure. The complexity of the response to DEB exposure is illustrated by the following example. DEB induces large amount of SCE in lymphocytes of individuals who are simultaneously GSTM1 null and CYP2E1 c1/c2. On the contrary, workers who have NAT2 G 590 allele have few SCE after DEB exposure. The amount of SCE is also different in function of EH polymorphism [137].

3.4.1.2. Implication of other polymorphisms (transporters; repair). Nevertheless, it should be kept in mind that genetic polymorphism could not alone explain all the inter-individual variations in the biotransformation and the impact of the xenobiotics.

In addition, activities of receptors and transporters driving distribution and elimination of xenobiotics are modulated by polymorphism (for a review, see [138]). This characteristic has repercussion on the side effect of chemicals. Modification in distribution and elimination could lead to accumulation of the toxin or one of its metabolites in target or non-target tissue, and thus can constitute a mobile reserve of the toxin.

In mammals, a complex system of repair allows to repair damage before replication. Polymorphism of these types of damage is essential in the carcinogenic

process. If the system repair is deficient, the individual is at higher risk for tumour development.

Studies concerning carcinogenic process and polymorphisms of repair enzymes are scattered. A review of Goode et al. [139] highlights implication of three polymorphisms of repair systems in the development of cancer: (i) variante OGG1 S326C (enzyme excising 8-OH-guanine) is linked with an increased risk of oesophagus, lung and prostate (ii) variant XRCC1 R194W (enzyme implicated in BER) is systematically associated to a decrease of bladder, lung, mammary and stomach cancer risk (iii) variant BRCA2 N372 H is systematically associated to an increase of mammary cancer risk.

3.4.1.2.1. XP enzymes. Significant higher amount of CA is observed in patients having the alleles (AA and AC) of XPD on exon 23. DNA single-break strand following sporadic exposures to chemical pollutant (such as cleaning compounds) at home or at workplace are dependent of XPD, XPG and XRCC1 polymorphisms [68,140,141]. The risk of lung adenocarcinoma is three times higher for individuals having the XPA AA [142].

A recent study has shown that coke-oven workers with a low repair ability due to the genotype XPC PAT +/+ and XPA-A23A have a significant higher amount of DNA adducts compared to workers who did not have these genotypes, or who have only one of these genotypes [43].

This difference is exemplified if in addition the workers are GSTM1 null. In this study, it is clearly established that the amount of BPDE-DNA adduct is proportional to the exposure and is significantly increased in workers who are GSTM1 null and for whom the repair ability was decreased by the genotype XPC-PAT +/+.

None of the three other polymorphisms of NER system (XPA-G23A; XPD-Asp312Asn and XPD-Lys751Gln) modulated the amount of BPDE-DNA adduct in coke-oven workers. In contrast, the genotype XPD-Gln751Gln alone [45] or combined to the genotype XPD-Asn312Asn lead to an increase of DNA adduct in smokers [44] or in policeman exposed to low amount of PAH [45].

3.4.1.2.2. XRCC enzymes. Individuals having the allele XRCC1-399Gln exhibited more Aflatoxin related DNA adducts [121] but also more SCE and DNA adduct related to polyphenol [143].

Coke oven workers having the halotype CGGG of XRCC1 gene are protected against chromosomal damage induced by PAH, whereas the halotypes TGGG and CGAG increase the risk [46].

Vinyl chloride induced formation of ethenobases leading to mutation of TP53 gene. This mutation induced an overexpression of the protein P53. This over-expression is correlated to vinyl chloride exposure. Nevertheless, after a similar exposure to vinyl chloride, workers heterozygotes Arg-Gln or homozygote Gln–Gln for the repair enzyme XRCC1 are more at risk compared to those who are homozygote Arg–Arg. The odd ratio (OR) is 1.73 (95% IC 0.93–3.22) and 3.95 (95% IC 1.68–9.28), respectively. If the individuals are in addition CYP2E1 c2, the risk is higher [56].

Exposure to styrene could be evaluated by the measurement of urinary mandelic acid. Another biomarker of styrene exposure is the amount of valine adduct on haemoglobin. This adduct induce DNA damage such breaks (which could be detected by comet assay), and micronuclei. Analyses of these parameters in 44 workers exposed to styrene compared to 44 unexposed workers shown (i) the relevance to these biomarkers and (ii) modulation of damages in function of repair enzymes polymorphisms. Thus, the occurrence of micronuclei in mononucleated lymphocyte (MNMC) was significantly higher in individuals having the allele XRCC3 Met231. Individuals having the allele XRCC1 Gln399 have the highest amount of both types of micronuclei, MNMC and MNBC (micronuclei in binucleated lymphocytes) [86].

3.4.2. What conclusions could be drawn from genotyping?

At population level, studies demonstrated that genetic factors drive the formation of reactive metabolites, and pinpointed the interest of biomarkers in relation to "exposure-potential carcinogenic risk", nevertheless a risk profile can not be drawn.

Studies from Ma et al. [144] and Teixeira et al. [145] underlined that genetic polymorphism and life style should be considered during the monitoring of workers exposed to styrene and that the sole measurement of metabolites in urine is not enough. Actually, for a same styrene exposure, less urinary metabolites are detected in urine of workers who have CYP2E1, CYP2B6 and EH with low activities. This is not correlated with a lower risk as higher amount of SCE, higher occurrence of DNA single breaks, chromosomal aberration and mutation of HPRT gene were observed in workers deficient in GSP1 and CYP2E1 [145]. Among the different biomarkers studied, most reliable seems to be DNA adducts detection, often allowing to identify the nature of the substance causing pathologies [146,147]. DNA adducts reflect cellular response to carcinogenic metabolites (and/or teratogenic) and are outcome of activation and repair processes [140,148]. This biomarker mirrors on one hand exposure and on the other hand the metabolic capacity of the individual. When the repair system is efficient, influence of genetic polymorphism of biotransforming enzyme is minimised and the carcinogenic risk is lower. Although it is not possible to eliminate all contamination sources, the knowledge of mechanism inducing cancer after exposure should allow defining individual and collective protections, favouring primary prevention (substitution of toxic by non-toxic compound; better work organisation …).

A genetic screening is not feasible at individual scale, because it will pose ethical, social and scientific problems. It should be kept in mind that the origin of a cancer is not the individual susceptibility but the fact that the individual is exposed to pollutants. Protection of workers with high susceptibility cannot be an alternative to the reduction of exposure to genotoxic agents at workplace. These studies on the role of genetic polymorphisms of biotransforming enzyme and repair enzymes should be conducted in the context of development of useful tools estimating the risk associated with exposure to genotoxic compounds with the aim to be able to reduce these exposures. Analysis of cytogenetic biomarkers could take place of the monitoring of exposed worker allowing protection of individuals, but should not be used for discrimination [149,150,151,152,153,154,155].

4. Time-Course Formation and Persistence of DNA Adduct — Is DNA Adduct Predictive of Cancer?

The development of carcinogen biomarkers to monitor human exposure and predict effects requires an understanding of the basic kinetics of response in a model that mimics the human exposure situation.

4.1. PAH

In rodent models, it can be clearly demonstrated that for a variety of polycyclic aromatic hydrocarbons, there is a quantitative relationship between the inductions of DNA adducts in target tissue DNA and tumour induction. In fact, if one measures DNA adducts at various times after a single administration of carcinogen, adduct levels increase over time, reach a maximum value and then decrease gradually over time. So the adduct level detected at any given time point will depend upon both the dose and the time when the measurement is made. If one measures adducts at multiple time points to establish the shape of the adduct persistence curve, then one can integrate the equation of the curve to determine the area under the curve. This area represents the total adduct burden experienced by the target tissue DNA over time from administration to tumour. It turns out that the potency of many different PAHs is well predicted by this single parameter.

Boerrigter et al. [156] have analysed the formation and disappearance of B(a)P-DNA adducts in six organs of two different mouse strains with different life spans, C57BL/6 and BALB/c. Maximum formation of the major BPDE-N2-dG adduct after a single, i.p. treatment, appeared to be organ- and age-dependent. The amount of DNA adduct was 2–8-fold lower in organs from old mice compared to young mice The removal of BPDE-N2dG up to 7 days after treatment was age- and strain-dependent but was similar from one organ to the other in each strain. In young C57BL/6, which have a greater life expectancy (700–600 days) than BALB/c (300–600 days), the rate of disappearance of BPDE-N2 dG adduct was significantly higher in three out of six organs (liver, lung and spleen) compared to young BALB/c mice. In contrast, at 18 months of age, the rate of BPDE-N2-dG adduct removal in organs from C57BL/6 was the same or even lower as compared to that found in organs from BALB/c mice. In another study, Talaska et al. [157] compared the kinetic of formation and removal of B(a)P-DNA adduct in skin after acute and chronic exposure. The rate of adduct accumulation with chronic topical exposure was tissue- and dose-specific. In skin target tissue, the total B(a)P DNA adduct increase linearly after treatment with 10 and 25 nM. The accumulation of adducts with the highest dose (50 nM) reaches a plateau after an initial early increase. The maximal adduct level was only two times higher following 30 twice weekly treatments compared to only one topical application. The amount of B(a)P DNA adduct was 10-fold higher in the primary target tissue (skin) than in an equivocal target tissue (lung). The non-target tissue (liver) exhibits two times less adducts than lung. Analysis of removal indicated that there is a compartmentalisation of damage and repair following exposure and that with chronic exposure an

accumulation of adducts occurs in a repair resistant compartment. The rate of DNA adduct removal was 2.5–8 times faster following a single dose than when animals were exposed for 15 weeks. The slope of removal was 7.6 and 1.3 adducts/10^9 nucleotides per hour, 12–24 h and 24–96 h. The slope of the last phase is consistent with removal of adducts based on a cell half-life of about 20 days [90]. Poirier et al. [158] also reported that there appeared to be two compartments of DNA in the liver; in one adducts were formed twice as fast and removed 13 times as fast as in the other. The benzo[g]chrysene is a pentacyclic PAH which occurs in coal tar and petroleum distillates, and is a skin carcinogen in mice. This compound induced the formation of four different adducts. The maximum of DNA adducts level was reached in 24 h after treatment. There was a loss of 58% of the damage by day four, followed by a slower removal of adducts over subsequent days. Each adduct followed the same pattern of removal. By 21 days, 13% of the total damage remained [159].

Following skin application of 90 mg/kg 5,9-dimethyldibenzo[c,g]carbazole (DMDBC), the level of DNA adducts increased sharply to a maximum at 24 h that was 20-fold higher than in the liver of mice treated with 10 mg/kg The number of DNA adducts then fell strongly at 144 h. At the same time, proliferation of cells was observed. Thus, the sharp decrease in the global DNA adduct level may partly result from cell proliferation and elimination of badly damaged cells instead of active DNA repair [160]. Such correlation has been also observed when mice were fed with coal tar [161]. The formation and removal of several PAHs over 8 days have been studied [162]. With complete carcinogens such as B(a)P and 5-methyl chrysene, the maximum of DNA adducts was reached in 24 h after treatment. The pattern of adduct loss follow a two-phase pattern with a rapid decrease between 24 h and 4 days, and a loss of more than 90% at day 7. This could be explained by DNA repair processes taking place in the first 2–3 days, followed by a gradual loss due to epidermal hyperplasia and desquamation after day 4. With initiator such as 1,4-dimethylphenanthrene and benz[a]anthracene, adducts took longer to peak (2 days), followed apparently by a decrease in adducts levels at day 4, suggesting that DNA repair takes place. However, this was followed by another increase in adduct levels. This could be due to failure to produce hyperplasia and/or to release of PAH accumulated in sebum onto skin surface. At low to moderate environmental exposure to carcinogenic PAHs, DNA adduct levels in WBC were significantly correlated with exposure. However at the higher occupational levels found on the coke oven, the exposure-DNA adduct relationship became non-linear [163]. This high exposure-dose non-linearity may be due to saturation of metabolic activation enzymes, induction of either DNA repair processes or detoxification enzymes. This decrease in the DNA adduct potency at moderate to high doses of PAH has important implications for dose-response extrapolation in risk assessment [164].

Several authors have used DNA adduct detection in aquatic organism to evaluate genotoxic impact after oil spill. Figures 21 and 22 show DNA adduct in liver of fish caught in front of ERIKA oil spill. Figure 21 presents typical liver DNA adduct pattern in sole sampled at four locations along the French Brittany coast — Perthuis (P); Bay of Bourgneuf (BB); estuary of Loire (EL); Vilaine South (VS) — and at three periods — February, July and September in the year 2000. Two different zones could be delimited on the autoradiogram: the diagonal radioactive

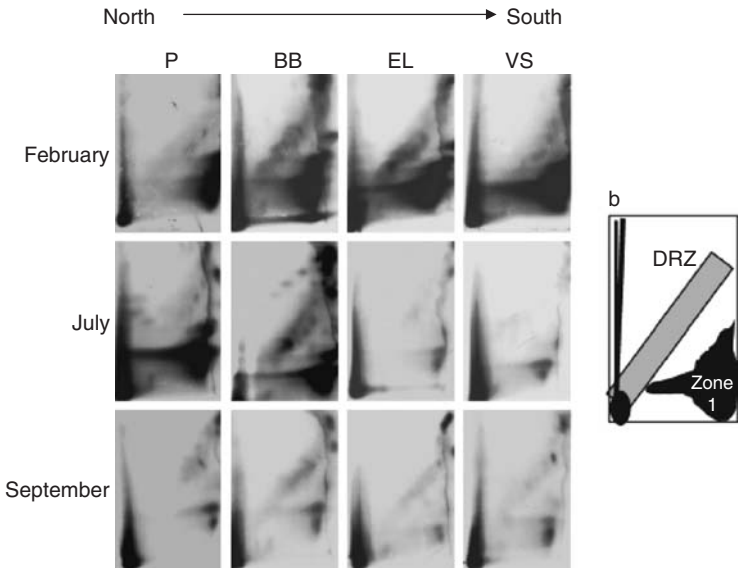


Figure 21 Typical autoradiograms of DNA adducts detected in fish liver after Erika oil spill.

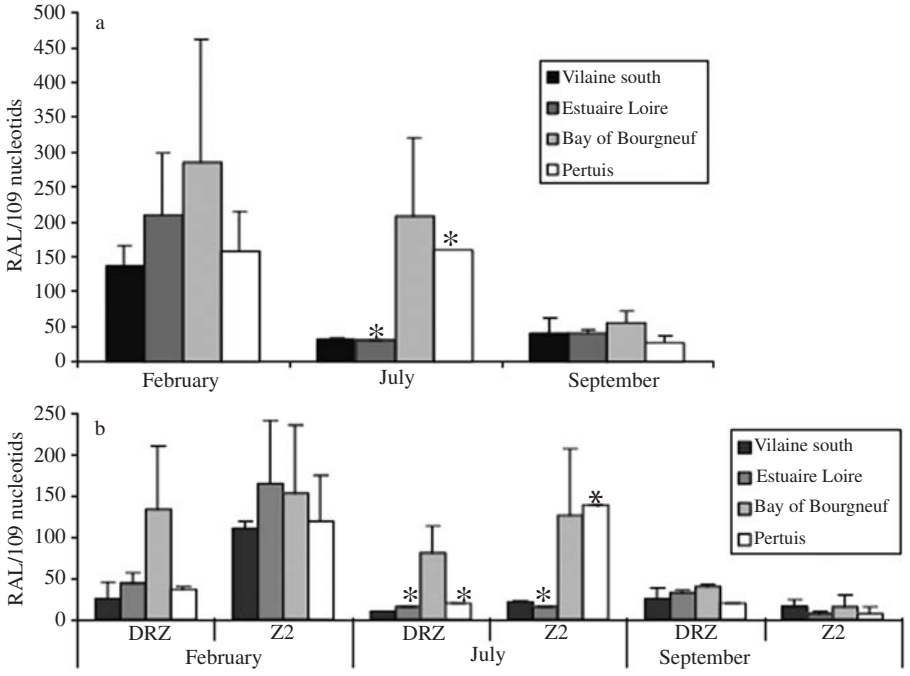


Figure 22 Amount of DNA adduct in liver of fish exposed to ERIKA fuel in marine environment (a) total DNA adduct expressed as number of adduct per 10^9 nucleotides (b) DNA adduct of DRZ and Zone 2.

zone (DRZ) and Zone 2. In February, DRZ was observed in all locations, even in Perthuis which is expected to be a non-contaminated region. Figure 20 gives the amount of DNA adduct and the differential removal of the DNA adducts. The highest total DNA adducts were observed in February, two months after oil spill whatever the location be. They considerably decreased in July in EL and VS about 80% [165,166]. Immediately after the oil spill (December 1999 and January 2000), a large amount of DNA adducts were observed in digestive glands of mussels also. From February to March, a dramatic decrease of DNA adducts in all the eleven sites was observed and could be explained by the depuration of the water probably due to the weathering and the cleaning operations. When we compare the presence of adducts with the relative genotoxic impact between sites (CRV values), it is possible to determine the two periods of genotoxic events: the first period from January to April–May 2000 and then in October 2000. The analysis of the chemical PAC contamination in mussels exposed to the Erika oil spill along the Atlantic coast has revealed the two same periods of chemical contamination which correspond to the direct chemical contamination by the oil spill from January to April–May 2000 and then the remobilisation of the pollutants in the environment in October 2000 [167].

Decline of PAH-DNA adducts in lymphocytes following smoking cessation suggests a half-life of 9–10 weeks [168]. Studies on lung DNA of ex-smokers have been conducted and revealed that DNA adducts could be detected five years after stopping [169,170]. However, it is unclear whether this has to do with slow removal of the DNA adducts, or is due to formation of adducts from residual PAH, which may remain present for many years in fat tissue. B(a)P and benzo[b]fluoranthene DNA adduct to blood lymphocytes have a half-live of 17–20 days [171,172], whereas ethylene oxide-DNA and methyl-DNA adducts in WBC have a half-live of only 2.9 and 1 day, respectively [173,174]. This type of profile was also observed with chronic dietary exposure to B(a)P of rat. The elimination was dose-dependent. DNA adduct accumulate in organ resistance to repair under chronic exposure. Moreover, as substances are lipophilic they are sequestered in lipid-rich tissues and serum proteins, and redistributed within the body even after cessation of exposure and thus DNA adduct remain relatively high [175].

A direct correlation between DNA adduct in healthy tissue and tumour is unlikely, because DNA adduct is the consequence of insult few days before formation, whereas tumour is formed several years after insult. However, what these studies do demonstrate is that exposures known to pose cancer risk-induced damage at the DNA in the target tissue, thus suggesting a causal relationship.

Lyons et al. [176] and Harvey et al. [177] who analyzed the genotoxic impact of Sea Empress crude extract on fish liver, and invertebrate species observed a persistent of few DNA adduct 12–17 months after oil spill in vertebrate but not in invertebrate animals. French et al. [178] observed a steady-state increase in DNA adduct level during a chronic exposure of English sole to PAH-contaminated sediment for 5 weeks and they are very persistent even after depuration period. In the same ways [179], they have also observed that hepatic DNA adduct formed in Atlantic code appeared 3 days after exposure to low concentration of crude

extract and was increasing steadily during the 30 days following exposure. Over 60% of DNA adducts remained for 60 days.

4.2. Heterocyclic aromatic amines

The formation and the removal of the isomeric dG-C8-IQ and dG-N2-IQ adducts were examined in rats and monkeys by ^{32}P-postlabelling. In these species, dG-C8-IQ, the main adduct formed following a single acute dose (10–20 mg/kg) was removed more rapidly than dG-N2-IQ in slowly dividing tissues such as liver, kidney, pancreas and heart. In contrast to these tissues, both adducts were removed at comparable rates in the colon, where the epithelial cells turn over rapidly [180]. In non-human primates, the level of dG-N2-IQ in the liver of chronically treated animals (20 mg/kg, 9 years) was approximately 3- to 5-fold greater than dG-C8-IQ. Moreover, the quantity of dG-N2-IQ increased by more than 100-fold over a single acute dose in slowly dividing extrahepatic tissue [129]. In monkeys that develop hepatocellular carcinoma after chronic feeding with IQ, dG-N2-IQ is the predominant adduct found in liver. However, this adduct is also found in non-target tissues (kidney, pancreas and heart), indicating that although DNA adduct formation after metabolic transformation after IQ contamination is needed, the relationship between DNA adduct and tumourigenesis is not a simple matter of cause and effect. The tumour develops after specific mutation in oncogenes inducing activation and in tumour-suppressor genes leading to inactivation [181]. MeQIx-DNA adducts level in liver increased in a linear dose-response manner in rats fed with various concentrations of MeQIx up to 400 ppm [182,183]. At 100, 200, 400 ppm, MeQIx, the incidence of hepatocellular carcinoma was 0, 45, 94%, respectively [184]. The removal of MeQIx-DNA adducts in liver of rats was reported to occur in a biphasic manner (for a review, see [181]). The formation and removal of PhiP have been tested in rat Fisher-344 (50 mg/kg, by gavage). The first day, total DNA adduct levels were highest in the colon (the target organ) followed by spleen, cecum, small intestine, stomach, liver, kidneys, lung, white blood cells and hearty. Rates of total DNA adduct removal of PhiP in male Fisher-344 rats (50 mg/kg) were similar in colon, spleen, cecum, liver, lungs, stomach and small intestine, with amounts at day 20 declining to less than 16% of those quantities found 1 day post-treatment [185]. There was no evidence for preferential removal of any specific adducts. Rate removals were slower in kidney and heart (30.3 and 52% of day 1 value remaining on day 16, respectively). Adduct in white blood cells increased at first (day 2) and decreased thereafter to virtually non-detectable levels on days 16 and 20. Heart adducts on days 2–12 increased slightly or remained as high as those on day 1, then decreased to lower amounts on day 16 and 20 (53 and 28.7% of day 1 quantities, respectively). The rate of DNA adduct removal from the intestines are more likely to be related to cell turnover of epithelial cells than to enzymatic repair [185]. Interestingly dG-C8-MeIQx was identified in 3/38 DNA samples of 13 individuals in colon, rectum and kidney. The DNA adducts ranged from 2 to 20 adducts per 10^{10} nucleotides. In 24 individual tissues samples including pancreas, colon and urinary bladder epithelium, dG-C8-PhiP was detected in only one-third of colon samples at levels of 2.9+0.5 adduct/10^8 nucleotides.

4.3. Ochratoxin A and aristolochic acid

Formation and persistence of specific DNA adduct related to aristolochic acid I have been studied in rat treated orally [186]. The two major adducts, 7-(deoxyadenosin-N6-yl)-aristolactam I (dA-AAI) and 7-(deoxyguanosine-N2-yl)-aristolactam I (dG-AAI) showed differential rates of removal and persistence, depending on the organ examined. In all target and non-target organs, dA-AAI was the predominant adduct over the entire duration of the experiment. In the target organ, forestomach, dA-AAI and dG-AAI adducts diminished rapidly within the first 2 weeks. dG-AAI was removed extensively, whereas dA-AAI was more persistent between 4 and 36 weeks after last exposure. A marked decrease of both adducts in glandular stomach was observed. Similar removal curves were observed in non-target tissues, namely lung and kidney. In contrast, both purine adducts were slowly removed from liver and urinary bladder [186]. Correlation between dA-AAI and mutation of Hras in forestomach has been established. Although AA is not carcinogenic for the kidney, it has been recently suggested that aristolochic acids could be involved in BEN and that dA-AAI could be found in kidney tumour several years after cessation of treatment. This hypothesis has been based on the fact that in Belgium some women have developed a similar nephropathy after being treated with slimming regimen suspected to contain AA [187]. The detection of specific AA-DNA adduct several months after cessation of treatment [188] even though that no AAs could be found in the pills [189,190], is considered as a relevant biomarker. As so long persistence of DNA adduct is questionable, we have analysed the formation and the persistence of DNA adducts in human kidney cells treated with increasing amount of either AAI or AAII or both of them, or in presence of OTA or CIT for 7, 24 and 48 h. In cell culture whatever the doses and the time of exposure are, only a small amount of AA related DNA adducts were formed in kidney cell culture, whereas a large amount of DNA adduct were formed in these cells after OTA or CIT treatment. All DNA adducts (related to AA or to OTA) have disappeared after 2 days. This indicates a fast repair of these adducts, whatever the kind of DNA adducts be, ruling out the possibility to find DNA adduct in kidney of women several years after cessation of contamination. This is in line with the fact that no adduct was found in the kidney tissues of the women treated with slimming regimen [112]. We have demonstrated that the preferential formation of either C-C8dGMP OTA or OTHQ-DNA adduct depends on expression of some biotransforming enzymes. Indeed, OTHQ-related DNA adduct were formed after *in vitro* incubation in presence of kidney microsomes of untreated pig or healthy human expressing mainly cyclooxygenase COX1 and CYP 2C9, whereas C-C8dGMP OTA was formed mainly after incubation in presence of kidney microsomes from pig fed with OTA or from human tumour, expressing mainly COX2 and lipooxygenase [109]. This is noteworthy that induction of COX2 often occurred during cancer process, notably in kidney. Incubation in presence of microsome from peri-tumoural part of human kidney has lead to the formation of the two OTHQ adducts in addition to C-C8 dGMP OTA [191] (for a review, see [110]). Comparison of DNA adduct in tumoural part versus peri-tumoural part confirmed that difference in metabolic capacity of the tissues lead to formation of

different types of adducts. More different DNA adducts are measured in peri-tumoural part and reached a higher level. In carcinogenic study we observed mainly C-C8dGMP OTA in tumour, and demonstrated that DNA adduct pattern was dependent of the metabolic capacity of the tissue [111,192]. Lower DNA adduct level observed in tumoural [112] part could be explained by the rapid proliferation of the tumoural cells inducing a dilution, and also by the disappearance of the DNA adduct due to accurate or not repair. Interestingly, OTA- and CIT-related DNA adducts are formed in range of nanomolar concentrations where the proliferation of kidney cells was observed. In contrast, treatment of cells with high dose of OTA decreased cell viability and lead to the formation of less DNA adduct. OTA induces different types of DNA adduct as a result of its metabolic transformation. The repair of the OTA-related adducts (OTHQ-related adduct and C-C8dGMP-OTA adduct) was differential. These data are in line of *in vivo* study, where we observed a fast repair of DNA adduct in liver and spleen, whereas some adduct persisted longer in kidney [193]. The persistent adduct were the same as DNA adduct found in kidney tumour of human beings suffering from BEN (for a review, see [110]). Unfortunately, we do not know which of the OTA-related DNA adducts are mutagenic. Recently, we have demonstrated that OTA cross the placenta and induces specific DNA adduct in pups. Nine months after birth, DNA adducts were found no more, but several months later the animals have developed renal carcinoma [191]. In contrast, specific DNA adduct related to AAs was not detected in any tumour analysed or in kidney of women having followed a slimming regimen [112].

4.4. Aromatic amines

Poirier and Beland [194,195] and Poirier [196,197] have reviewed the data from separate experiments with five different carcinogens with respect to tumour induction by chronic lifetime exposure in a broad dose-range, along with those of analogous studies in which DNA adducts have been a measure in target tissues of rodents exposed to similarly for 1–2 months. The compounds studied included 2-acetylaminofluorene (2-AAF), 4-aminobiphenyl (ABP), aflatoxin B1, diethylnitrosamine, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK). Different types of relationships between dose and DNA adducts, and dose and tumourigenesis were observed (Figure 22). In the simplest situation, both DNA adducts and tumours increased linearly with dose (case A with 2-AAF in liver of female mice, and AFB1 in liver of male rat). Sometimes, although DNA adduct levels and tumour were linear with dose, the tumour incidence never reached 20% (case B). This was the case with 4-ABP in male mouse liver and female mouse bladder. This implies the existence of effective detoxification pathways that may reduce activation to DNA adduct formation. In case C (4-ABP in liver of female mice, DEN liver of male rat; NNK in lung of male rat), the profiles for DNA adduct and tumour incidences to be linear at the lower doses, but failed to increase proportionally with dose at the highest doses. The saturation of DNA adduct formation is due to a saturation of metabolic processes. In addition, both compounds induce cytotoxicity that suppressed DNA adducts formation and tumourigenesis. In the last case (case D; AAF in bladder of female mouse; ABP in bladder of male mice) DNA

adduct formation was linear, but tumour appeared only for high doses. This steep increase of tumour was correlated with cell proliferation. Thus, DNA adducts in bladder appeared to be necessary for tumourigenesis but were not sufficient until the bladder cell proliferation rate reached a critical level.

5. Conclusion

The formation of DNA adducts is believed to be an essential step in the carcinogenic process. It has been established for a number of carcinogens that persistent DNA adducts (those that are repaired more slowly) are more closely related to carcinogenic potency than are non-persistent adducts.

DNA adducts are frequently used as biomarkers of exposure to chemicals that are either electrophilic or are metabolised to electrophiles [198,199,200,201]. This is readily possible in experimental systems with known exposure and duration, but it is a much more complex issue in molecular epidemiological studies [202,203,204, 205,206]. Experimental studies have shown that with constant dosing, a steady-state concentration of DNA adduct will occur, where the number of new adducts formed each day equals the number of adducts that are lost due to repair. Thus if the exposure is relatively constant in a molecular epidemiological study, it can be reasonably assumed that adducts are at a steady-state concentration. In contrast, if exposures are intermittent and of unknown and variable amount, little inference can be made other than that exposure occurred. Many different DNA adducts are now being measured, but a major issue in interpretation relates to their meaning [207,208,209, 210,211,212]. The presence of a chemical-specific DNA adduct in human DNA is a good indication that exposure to that chemical occurred [213]. Carcinogen-DNA adducts in target tissues are a more relevant marker than internal dose, because the former reflect not only individual differences in absorption and distribution, but also difference in the metabolism (activation versus detoxification) and the extent of repair of DNA damage. DNA adduct data are useful to clarify carcinogenicity results to elucidate the mechanism of carcinogenesis or to evaluate low dose-range effects.

Lower DNA adduct level observed in tumoural part could be explained by the rapid proliferation of the tumoural cells inducing a dilution, but also by the disappearance of the DNA adduct due to accurate or not repair. DNA adducts have vastly different potentials for causing mutations. Some adducts are highly mutagenic, whereas others are not mutagenic and do not lead to heritable effects [214]. In the latter case, the presence of such adducts is simply a measure of exposure.

Under conditions of steady-state exposure, the levels of chemical-specific DNA adducts may be expected to reflect the biologically significant individual exposure to specific agents which in turn could be expected to correlate with risk as exemplified by the adducts of aflatoxin B1 [215]. Thus the measurement of DNA adduct in target tissue has the potential to be not only an exposure marker, but also an individual cancer risk marker (for a review, see [216]). In most cases, the DNA adducts that lead to initiating events in inducing a tumour are removed from the tissue many years in advance of tumour growth and detection [217]. This explains the lower level of DNA adduct in tumoural part. So the adducts in the tumour have

perhaps nothing to do with the DNA adducts that induced the tumour. Nevertheless, the presence indicates clearly an exposure. Persistence of an adduct *in vivo* will depend on a number of factors, namely its inherent chemical stability, whether any active repair are present and the turnover of the macromolecule to which the chemical is bound. The parameter of persistence is particularly critical in determining the appropriate sampling times in epidemiological studies. In case of DNA adducts, the chemical stability is highly variable. For example, the AFB1-N7-dG adduct is lost by depurination ($T_{1/2}$ about 8 h in rat liver), while a proportion of the initial DNA adduct persist as the AFB1-Fapy in rat liver DNA for as long as 19 weeks after the last treatment [218]. Persistence of DNA adduct is also dependent on the stability of the molecule to which the chemical is bound. In the case of DNA, this is essentially translated as the rate of cell turnover. Thus, DNA adducts in epithelial cells from intestine or in neutrophils in peripheral blood ($T_{1/2}$ about 8 h) [129,219] will be far less persistent than adducts in non-proliferating tissues such as brain or liver. One would predict that DNA adducts amounts in the same individual should be higher in lymphocytes than total leukocytes. Altogether DNA adduct detection are useful to clarify carcinogenicity results, to elucidate the mechanism of carcinogenesis or to evaluate low dose-range [220,221,222,223,224] effects (Figure 23).

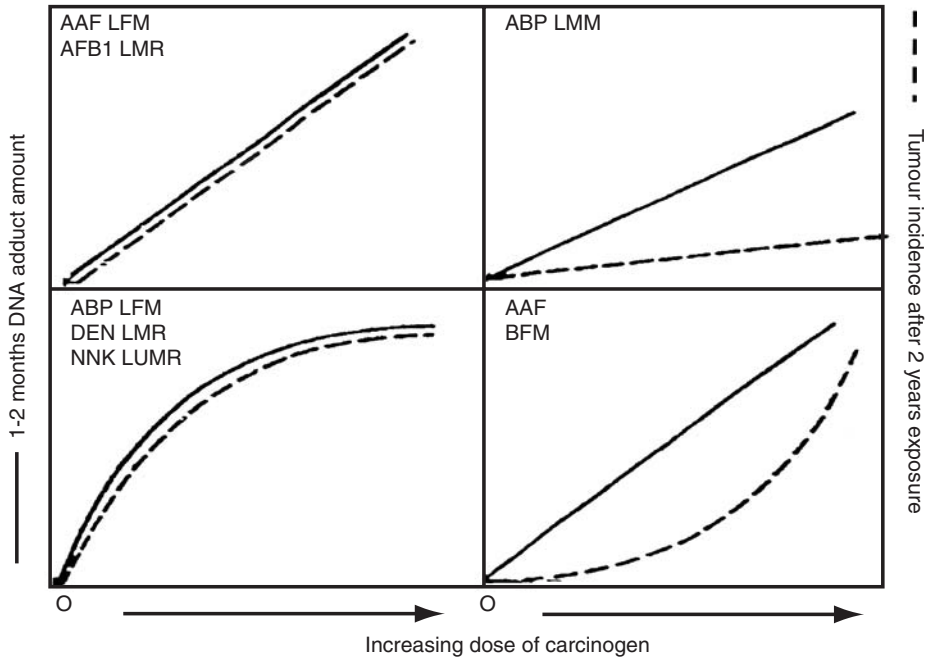


Figure 23 Relation between DNA adduct and tumour development. AAF, acetylaminofluorene; AFBI, aflatoxin B1; ABP, aminobiphenyl; DEN, diethylnitrosurea; NNK 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone; LFM, liver female mice; LMM, liver male mice; LMR, liver male rate; LUMR, lung male rate; BFM, urinary bladder female mice; BMM, urinary bladder male mice.

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