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Pharmacological and Toxicological Considerations of Homogentisic Acid in Alkaptonuria

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Abstract: Background: Patients with alkaptonuria are exposed to a high body-burden of homogentisic acid, due to a genetic deficiency of the enzyme homogentisate 1,2-dioxygenase. Younger patients appear to be more robust at withstanding or adapting to this chemical stress. As the patients age, the deposition of ochronotic pigments appears in connective tissues. This is thought to lead to the weakening of tissues and development of osteoarthritis. Currently, there is no marketed drug to treat alkaptonuria and the disease is managed through non-steroidal anti-inflammatory agents. **Results:** This review considers potential pharmacological strategies for increasing the excretion of homogentisic acid, with the goal of reducing the exposure of patient tissues to this acid. In order to define these strategies, we need to understand the processes by which low molecular weight compounds are handled by the body and enzymatically manipulated into forms which are more easily excreted. These processes are more commonly referred to as drug metabolism and are well understood within the pharmaceutical industry, who are required to design drugs that can reach pharmacological targets at a concentration required to achieve efficacy. Drug metabolism consists of phase I and phase II reactions. Phase I reactions (oxidation, reduction or hydrolysis) are required to increase the chemical reactivity of a molecule by adding or uncovering a more reactive chemical group. Phase II reactions add a polar, hydrophilic conjugate molecule (glucuronide, sulphate, GSH), increasing the renal excretion. In certain cases, drug metabolism can produce reactive and potentially toxic metabolites which can bind to DNA leading to carcinogenesis, or bind to protein leading to either direct organ toxicity (e.g., paracetamol) or immune-mediated toxicity (e.g., halothane). **Conclusion:** A better understanding of these processes within a disease such as alkaptonuria will help design rational patient management strategies through increasing the excretion and reducing the overall body burden of homogentisic acid.

Key words: Homogentisic acid, alkaptonuria, ochronotic pigments, drug toxicity

INTRODUCTION

Patients with the genetic deficiency, alkaptonuria, are unable to complete the catabolism of tyrosine/phenylalanine. Deficiency of the hepatic and renal enzyme, homogentisate 1,2-dioxygenase, results in accumulation of Homogentisic Acid (HGA) which is then excreted in large quantities in the urine (Phornphutkul *et al*., 2002). In infants, alkaptonuria, appears clinically relatively benign and is normally first observed through the production of black urine observed in the stained nappy/diapers. However, as the patient ages, adverse manifestations include ochronosis (dark staining of skin and connective tissues), chemical osteoarthritis of synovial joints and degenerative spinal disc disease can occur along with stone disease and aortic valve damage (Phornphutkul *et al*., 2002; Suwannarat *et al*., 2005; Helliwell *et al*., 2008).

HGA has a high renal clearance (400-500 mL min^{-1}) leading to very high urinary excretion (Phornphutkul *et al*., 2002). However, relatively little is known about the clearance mechanisms underlying excretion of homogentisic acid, as many publications investigating alkaptonuria simply measure HGA excretion without taking its metabolite(s) into account. HGA can undergo both spontaneous and enzymatically-mediated oxidation to a more chemically reactive species (Zannoni *et al*., 1969; Wolff *et al*., 1989), Benzoquinone Acetic acid (BQA). BQA can bind to proteins (Stoner and Blivaiss, 1967) with the additional formation of reactive oxygen species and free radicals (Martin and Batkoff, 1987). This in turn is thought to be responsible for the dark colour or ochronotic pigment which stains connective tissue. However, this can also form in basic solutions of HGA *in vitro*. Reduction of the BQA back to HGA can also occur, making any assessment of HGA

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levels at best, a 'snap-shot' of a dynamic equilibrium. If we consider the *in vivo* situation, excluding the kidney, the predominant organ responsible for the abnormal accumulation of HGA, the liver, contains all the enzymes, such as cytochrome P450's and transferases, required to facilitate excretion of low molecular weight compounds. This type of consideration is required within the pharmaceutical industry in drug design and drug development. Compounds entering the liver, if they undergo considerable 'first-pass' hepatic metabolism are unlikely to reach their target at a sufficient concentration to elicit efficacy. *In vitro* studies with translation to *in vivo* efficacy and considerable Pharmacokinetic (PK) modelling, has to be undertaken to ensure the development of a sufficiently effective lead compound. Indeed, in 1991 undesirable PK and bioavailability characteristics were responsible for the failure or attrition of nearly 40% of drug development. However, in 2000, with these processes becoming much better understood, this failure rate diminished to less than 10% (Kola and Landis, 2004). Further considerations of non-clinical drug safety also need to be taken into account, so drugs also undergo considerable toxicological screening. The assessment of chemical liability is undertaken, i.e., does the chemistry of the drug contain a structure that has the potential for hazard at a later stage. This could be either in the development pipeline or once marketed and taken by a diverse population of patients. The nature of the potential hazard also has to be identified, then removed or re-designed from within the drug structure. For example, this hazard could take the form of genotoxicity, drug-drug interactions, cytochrome P450 inhibition, transporter protein inhibition, hERG inhibition (QT prolongation), reactive metabolite formation etc, all of which can cause problems later in development.

The aim of this review is to consider HGA as a drug from a PK and toxicological or drug safety perspective. Similar to the drug safety principles used in the pharmaceutical industry, can we assess the hazard posed by HGA and its metabolite(s). However, in contrast to the pharmaceutical industry, with further knowledge of the excretion route of HGA, can we facilitate its more rapid elimination from patients suffering from alkaptonuria. In order to do this, an understanding of the principles of drug metabolism is required, along with the consideration of how drug metabolism can lead to toxicities and adverse reactions in animals and patients.

THE NORMAL PROCESS OF DRUG METABOLISM

The liver is quantitatively the most important site of metabolism of chemicals and drugs in the body (Park *et al*., 2005). The process of drug metabolism is of central importance in determining the duration of action, pharmacological effect and toxicity of drugs. In general, it can be regarded as a detoxification mechanism in that it facilitates the excretion of drugs and foreign compounds from the body by converting them from lipid soluble, non-polar compounds to more polar, hydrophilic compounds. The molecular changes produced by the drug metabolising enzymes almost invariably increase the rate of elimination of a drug and usually result in a loss of pharmacological activity. However, in certain circumstances, normal metabolic biotransformations can result in the formation of biologically active molecules which account, either in whole or in part, for the pharmacological and toxicological actions of the parent compound (Park *et al*., 1998). Drug metabolising processes are often divided into two phases, termed phase I and phase II reactions (Williams, 1971). These reactions are catalysed by a wide range of enzymes found in various sub-cellular locations, such as the mitochondria, cytosol and endoplasmic reticulum. For the purposes of this review, the term 'drug metabolism' encompasses the enzymatic biotransformations of all low molecular weight chemicals (endogenous and exogenous) that facilitates their excretion.

PHASE I AND PHASE II METABOLISM

When chemicals and drugs are absorbed from the gastrointestinal tract, they enter the hepatic portal vein which transports them to the liver. Within the hepatocytes of the liver, drugs are exposed to the enzymes which facilitate Phase I and Phase II metabolism.

The phase I biotransformations include oxidative, reductive and hydrolytic reactions. The major enzyme system involved in oxidative drug metabolism is the cytochrome P450 mixed function oxidase system (Fig. 1). This is responsible for approximately 95% of phase I metabolism and located predominantly in the smooth endoplasmic reticulum. These enzymes are mainly present in the liver but are also found in the kidney and gastrointestinal epithelium (Woolf and Jordan, 1987). The cytochrome P450 enzymes are able to catalyse the biotransformation of many chemically and biologically unrelated substrates (Gonzalez, 1991). Indeed, they have been described as the most versatile biological catalyst known. The P450 catalytic cycle involves activation of oxygen rather than binding of a substrate; therefore, the metabolism of a wide range of low molecular weight lipophilic molecules is possible. They are a superfamily of over 200 haem-thiolate enzymes which are encoded by multiple genes (Gelboin *et al*., 1990; Gonzalez *et al*., 1990). Characteristics of the enzymes include a non-covalently bound haem group with a strongly conserved binding site

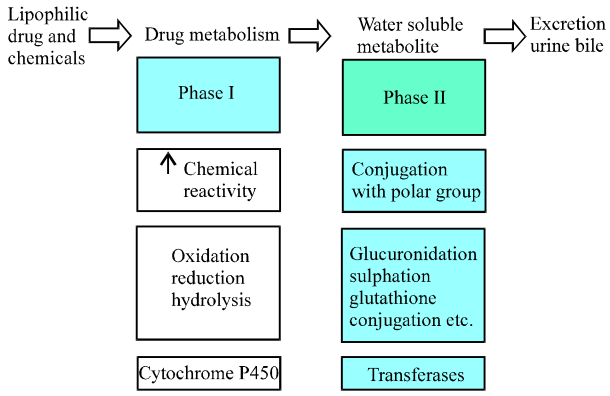

Fig. 1: General overview of the phases of drug metabolism

and a relatively low substrate affinity combined with a powerful oxidising capacity.

Phase II metabolic pathways include conjugation reactions to form glucuronide, sulphate ester, glutathione (GSH; Gly-Cys-Glu) and mercapturic acid, amino acid, methyl and acetyl conjugates (Woolf and Jordan, 1987). A drug may undergo sequential phase I and phase II reactions before excretion (Fig. 1), or alternatively, it may be conjugated without having to undergo functionalisation if a suitable functional group is available on the parent compound (Tephly and Burchell, 1990). Phase II reactions, like phase I reactions, can result in the formation of active metabolites which may cause toxicity (Pirmohamed *et al*., 1994). Quantitatively, the most important of the phase II reactions is glucuronidation, possibly due to the relative abundance of the Uridine Diphosphate Glucuronic Acid (UDPGA) cofactor which is found in all tissues of the body. The reaction is catalysed by a family of glucuronyltransferases which have a very broad substrate specificity (Burchell *et al*., 1991). Conjugation involves the transfer of glucuronic acid from the high-energy UDPGA to an electron rich atom (O, N or S) on the substrate (alcohols, phenols, hydroxylamines, carboxylic acids, amides, sulphonamides and thiols). Sulphation, catalysed by sulphotransferases, is a major conjugation pathway for phenols, alcohols, amines and to a lesser extent, thiols. Many aromatic amines undergo acetylation and amino acid conjugation, the former being catalysed by the polymorphically expressed N-acetyl transferase family (Jordan and Woolf, 1987).

Conjugation with the tripeptide GSH is recognised as an important protective system for removing potentially toxic electrophilic metabolites. GSH is the most important intracellular thiol (Larsson *et al*., 1983), reaching millimolar concentrations in most cells. Conjugation with GSH can occur either spontaneously or may be catalysed by a family of glutathione S-transferases, each of which have unique but overlapping substrate specificities (Di Pietro *et al*., 2010). The spontaneous reaction is more likely to occur with the so-called 'soft' electrophiles while enzymatic catalysis is more common with the 'hard' electrophiles. Enzymatic catalysis assumes dominance when the GSH concentrations are low, even with soft electrophiles (Coles *et al*., 1988). Compounds that can conjugate to GSH include epoxides, quinoneimines, aliphatic and aromatic halo- and nitro-compounds and alkenes.

RELATIONSHIP BETWEEN DRUG METABOLISM AND DRUG TOXICITY

Drug toxicity in man is dependent upon both the rate of metabolism which will influence the amount of drug/metabolite present at its site of action and the route of metabolism which will determine the nature of the chemical entities present in the body. Where the parent drug is responsible for the tissue damage observed, individuals unable to metabolise the drug will be at greater risk of toxicity if metabolic clearance represents a substantial proportion of the total body clearance (Park *et al*., 1998). Drug metabolism may become impaired by drug-drug interactions, viral infection and hepatic disease, or there may be a genetic component. The possibility of toxicity, when a drug metabolite is involved is determined by the relative rates of bioactivation and detoxification. These rates are of crucial importance in the case of short-lived chemically reactive metabolites in determining cellular viability (Park *et al*., 1998).

In certain circumstances, dependent upon the chemistry of the drug and the site of metabolism, oxidative biotransformation may lead to the formation of chemically reactive metabolites that can interact with various cellular macromolecules such as nucleic acids and proteins resulting in various forms of toxicity. Theoretically, such

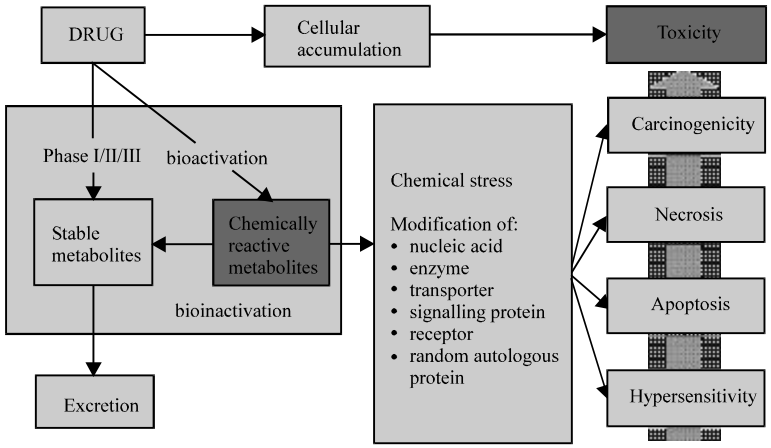

Fig. 2: How drug metabolism can lead to toxicity. Drugs can accumulate directly leading to toxicity. Drugs can undergo enzymatic reactions which lead to increased reactivity or electrophilicity of the compound. In general these compounds are detoxified. In certain cases they can interact with cellular proteins or DNA leading to an adverse drug reaction

reactive metabolites could cause direct apoptosis/necrosis, hypersensitivity, tetratogenicity and carcinogenicity (Fig. 2) (Park *et al*., 1998). In addition, the original insult may be subject to a degree of 'biological amplification', thereby overwhelming tissue repair processes causing extensive damage. This can arise when the toxic metabolite initiates chain reactions, futile oxidation-reduction cycles, an immune response, or elicits a series of viable cell mutations (Park *et al*., 1998).

When drug bioactivation is tightly coupled to bioinactivation then the process is one of physiological clearance. If these processes become uncoupled then the reactive metabolite may undergo chemical reactions with cellular proteins, lipids and nucleic acids, leading to protein dysfunction, lipid peroxidation, DNA damage and oxidative stress (Fig. 2). Additionally, the metabolites may induce disruption of ionic gradients and intracellular calcium stores, resulting in mitochondrial dysfunction and loss of energy production. This impairment of cellular function can result in cell death and possible organ failure (Williams *et al*., 2002; Williams and Park, 2003; Park *et al*., 2005). Electrophilic reactive metabolites can react spontaneously with GSH or may require a transferase to effect conjugation. The route followed by an electrophile may be concentration dependent e.g., N-acetyl-p-benzoquinone imine (NAPQI) may react spontaneously with GSH at physiological concentrations but requires a transferase, via glutathione-S-transferase enzymes (GST) at low concentrations of the endogenous nucleophile. Indeed, the conjugation to cellular antioxidants, such as GSH, represents the first level of defence adopted by the cell to the uncoupling of bioactivation/bioinactivation. The ability of a chemical to be bioactivated can be readily determined either by surrogate assays which measure the potential of drugs to become irreversibly bound to protein either *in vitro* or in animal studies or by mass spectrometric detection of thioether (e.g., GSH) conjugates. However, covalent binding and thioether adduct formation experiments only inform the chemistry (risk) of the molecule and cannot be used to predict biological outcome (hazard).

Cells have efficient cell defence systems to detect chemically reactive species and any modifications they may cause prior to cell death. An array of transcription factors and signalling proteins that have been implicated in sensing chemical stress and potentially driving adaptation e.g., AP-1, NF-κB, Nrf2, PPAR-gamma and other nuclear receptors, have evolved within the mammalian cell to add additional layers of defence. However, it is clear that no single pathway acts as the ultimate sole determinant of adaptation. Nevertheless, induction of several transcription factor pathways, such as Nrf2, may be a mechanism for adaptation to chemical toxicity and through reactivity of key cysteine residues in the inhibitor of Nrf2, Keap1; this pathway can 'sense' chemical danger and orchestrate cell defence (Copple *et al*., 2010).

HEPATOTOXICITY DUE TO THE METABOLISM OF PARACETAMOL

Paracetamol (acetaminophen; N-Acetyl-Para-Amino Phenol; APAP) is a major cause of drug-related morbidity and mortality in humans, producing massive hepatic

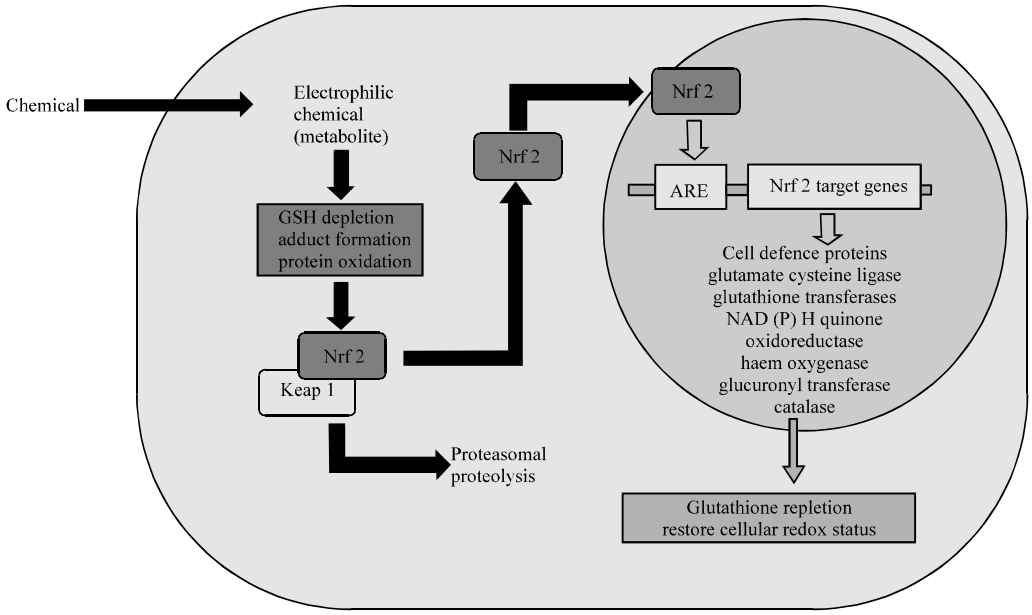

Fig. 3: The Nrf2 system helps cells adapt to chemical stress. When electrophiles are formed within cells, they can be 'sensed' by redox sensitive proteins, such as Keap-1. When thiol groups in Keap-1 become oxidised or conjugated with electrophilic agents, Nrf2 becomes dissociated from Keap-1. Nrf2 translocates to the nucleus and can bind to the Antioxidant Response Element (ARE) in the promoter region of many cell defence genes. This process is now well recognised to result in the up-regulation of many cell defence proteins which are able to restore the antioxidant balance leading to cellular adaptation

necrosis after a single toxic dose. At therapeutic doses, APAP is safely cleared by glucuronylation and sulphation to metabolites that are rapidly excreted in urine. However, a proportion of the drug undergoes bioactivation to N-Acetyl-Para-Quinoneimine (NAPQI). NAPQI is rapidly quenched by a spontaneous and enzymatic reaction (Coles *et al*., 1988) with hepatic GSH after a therapeutic dose of APAP (Henderson *et al*., 2000). However, after a toxic (over) dose, GSH depletion occurs which is an obligatory step for covalent binding and toxicity (Davis *et al*., 1974).

The massive chemical stress mediated by an APAP overdose leads to an immediate adaptive defense response in the hepatocyte. This involves various mechanisms, including the nuclear translocation of redox-sensitive transcription factors such as Nrf-2 (Fig. 3) which sense oxidative stress and orchestrate cell defense. The transcription factors and signalling proteins that have been implicated in sensing and potentially adapting to chemical stress with associated endogenous perturbation, upon exposure to acetaminophen or other xenobiotics is extensive, for a review see (Copple *et al*., 2010). Importantly, it has been observed that nuclear translocation occurs at non-toxic doses of acetaminophen and at time-points before overt toxicity is observed (Goldring *et al*., 2004). Specific knockout of the Keap1 gene in mouse hepatocytes confers a strong resistance to drug-induced toxicity (Okawa *et al*., 2006). This would indicate that the constitutive activation of Nrf2 and concomitantly its target genes is advantageous for mice to overcome xenobiotic toxicity. During the past ten years, the Keap1-Nrf2-ARE signalling pathway has emerged as an important regulator of the mammalian defence system enabling adaptation to chemical and oxidative stress through the induction of phase II detoxifying enzymes and antioxidant proteins (Prestera *et al*., 1993; Buetler *et al*., 1995; Hayes and McMahon, 2001; Lee and Johnson, 2004). Under normal physiological conditions, Nrf2 resides in the cell cytoplasm where it associates with a repressor protein, Keap1 (Itoh *et al*., 1999). Upon disruption of this interaction, Nrf2 is released and migrates to the nucleus where it can bind to the Antioxidant Response Element (ARE) present in the promoter regions of many stress-activated genes, such as HO-1, quinone oxidoreductase (NQO1) and GCLC (Venugopal and Jaiswal, 1996; Venugopal and Jaiswal, 1998; Alam *et al*., 1999; Wild *et al*., 1999) as part of a heterodimeric complex with other nuclear proteins (Fig. 3). Keap1 is known to play an active role in Nrf2 regulation by directing it for proteasomal proteolysis

(Itoh *et al*., 2003; McMahon *et al*., 2003). This indicates that the cell is primed to respond to a chemical insult through rapid up-regulation of Nrf2-driven defence proteins in a situation analogous to the action of p53 (Harris and Levine, 2005). Hence, the critical step in initiating a phase II response is the perturbation of the interaction between Keap1 and Nrf2; this is supported by the enhanced Nrf2 activity seen in Keap1 null transgenic mice (Wakabayashi *et al*., 2003) and in cells transfected with Keap1-specific siRNA (Devling *et al*., 2005).

THE RELATIONSHIP BETWEEN COVALENT BINDING AND TOXICITY

The bioactivation and covalent binding of drugs has been linked to cases of toxicity with numerous compounds. However, even when a drug forms protein adducts it does not necessarily lead to toxicity. There are several examples of structurally similar drugs that covalently bind to protein and yet have contrasting toxicity outcomes. These examples show that covalent binding should not alone be taken as an indicator of toxicity, daily dose and the target protein must also be taken into account. Nevertheless, in the case of APAP, blocking covalent binding by either chemical, biochemical or molecular interventions simultaneously blocks hepatotoxicity.

APAP is a model hepatotoxin however, its regio-isomer N-acetyl-m-aminophenol (AMAP) does not produce liver toxicity (Roberts *et al*., 1990). APAP is metabolised to the quinone-imine NAPQI which has been identified as the metabolite responsible for covalent binding. AMAP is metabolised to several quinone derivatives that are also capable of binding to protein. It has been demonstrated that it binds to protein at a similar extent to APAP. The comparable binding of APAP and AMAP, yet differing toxicity, has led to the analysis of the proteins that APAP and AMAP react with. Whilst AMAP binds mainly to cytosolic and microsomal proteins, APAP binds at a much higher level to mitochondrial proteins (Myers *et al*., 1995; Matthews *et al*., 1997). APAP is known to cause mitochondrial damage leading to cell death. In contrast, AMAP has been shown not to cause mitochondrial damage. This suggests that the amount of covalent binding may not be as important as the site of protein modification.

The metabolic activation of compounds and the covalent modification of cellular, sub-cellular and blood plasma proteins by reactive metabolites, in general, has received considerable attention (Yan and Caldwell, 2003; Kalgutkar and Soglia, 2005; Park *et al*., 2005). Nevertheless, the structure of the metabolite-protein adduct has been determined in only a few cases and identification of the modified amino acid residue (s) *in vivo* remains a major analytical challenge. Greater progress has been made in identification of the cellular proteins that are modified *in vivo* (Qiu *et al*., 1998; Shipkova *et al*., 2004; Druckova *et al*., 2007; Koen *et al*., 2007; Ikehata *et al*., 2008). Potential targets within individual organelles can now be identified by using model electrophiles in cell fractions (Shin *et al*., 2007; Wong and Liebler, 2008). This approach has the potential to confirm the association of selective modification of liver proteins with loss of critical enzyme activities (Campian *et al*., 2002) suggested by various studies on APAP (Park *et al*., 1998). The extent to which a particular loss of activity *in vivo* is due to adduction by a reactive metabolite or drug-induced oxidative modifications of amino acid residues is a complex analytical problem (Andringa *et al*., 2008). Despite these difficulties, the expectation is that a comprehensive database of cellular proteins modified covalently by reactive metabolites *in vivo* should ultimately facilitate elucidation of the link between reactive metabolites and associated pathologies (Hanzlik *et al*., 2007). It is already apparent that although each bioactivated xenobiotic may modify a unique set of hepatic proteins, there is a partial commonality with the proteins modified by other compounds (Koen *et al*., 2007). A similar selectivity is seen with model electrophiles *in vitro* (Shin *et al*., 2007). The proteins modified perform a great variety of biological functions, with corresponding potential for disruption of those functions. The relationship of the adduction of these proteins to the toxicity of reactive metabolites has been discussed in terms of inhibition of enzymes critical to maintenance of cellular energy and homeostasis, the unfolded protein response and interference with kinase-based signaling pathways (Ikehata *et al*., 2008). The targeting of components of signaling pathways and metabolic networks has been proposed, alongside the need to understand mechanisms of damage at a systems level (Liebler, 2008). In addition to consideration of the total amount and site of covalent modification, one should consider the rate of binding between specific reactive metabolites and their targets, a proposal originally suggested by Gillette (1974a, b).

THE METABOLISM, OXIDATION AND TOXICITY OF HOMOGENTISIC ACID

Further metabolism of HGA: There have been very few studies that have investigated the further metabolic routes available to HGA other than the oxidation to BQA.

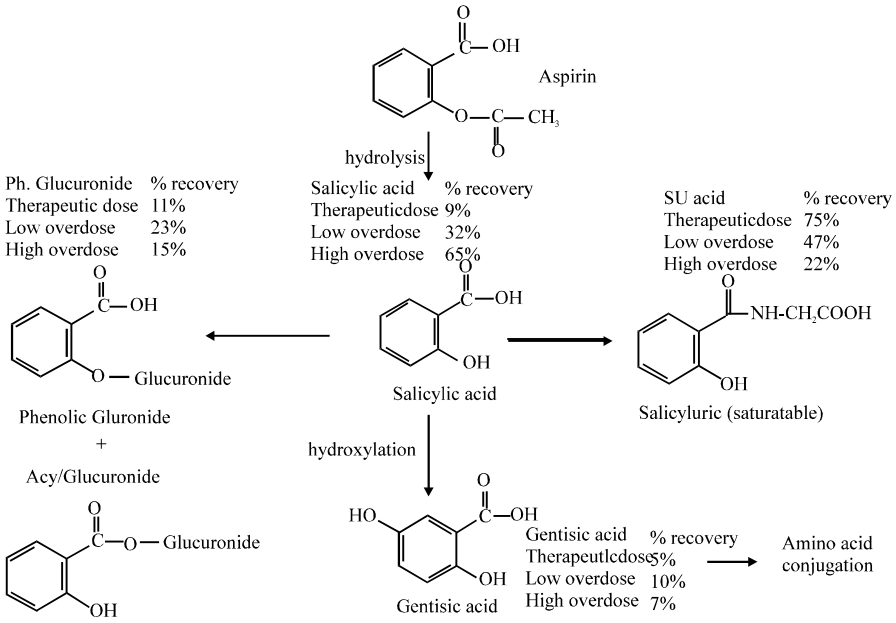

Fig. 4: The fractional clearance of aspirin during normal therapeutic doses and overdose cases. This slide (adapted from (Patel *et al*., 1990)) demonstrates that in aspirin overdose, the clearance levels of salicyluric acid (conjugate of salicylic acid and glycine) are greatly reduced. This indicates that the pool of hepatic glycine maybe a limiting factor of the efficient clearance of aspirin

This is probably due to the high renal clearance of HGA and the assumption that most HGA is cleared via this pathway. The tyrosine catabolic pathway resulting in the formation of homogentisic acid occurs predominantly in the liver, specifically, in the cytosol of hepatocytes. This location places HGA formation in close proximity to many enzymatic processes which could further metabolise (Phase I /Phase II) HGA. It seems pertinent at this stage, due to the lack of papers directly assessing metabolism of HGA, to compare the metabolism of HGA with that of a very similar weak, organic acid, acetylsalicylic acid (aspirin).

There are many studies on the metabolism and excretion of aspirin, however, an interesting study compares the metabolism of aspirin after both therapeutic and toxic doses (Patel *et al*., 1990). This is of more relevance to alkaptonuria as the levels of HGA produced are more akin to that observed in an overdose situation. Patel *et al*. (1990), studied the metabolism and excretion of aspirin in 45 volunteers who were given a therapeutic dose (600 mg) of aspirin alongside patients who had taken overdoses. The level of aspirin taken was quantified based upon the plasma levels of salicyluric acid (SUA). Twenty four patients had SUA plasma levels of between 240-600 mg L^{-1} and 13 had SUA plasma levels of 715-870 mg L^{-1}. It was found that there was reduced excretion of salicylate as SUA and this was accompanied by increased elimination of gentisic acid (metabolite of aspirin, not HGA) and salicylic acid phenolic glucuronide. This indicates that the unsaturated processes which lead to the formation of these metabolites significantly contribute to the inactivation of large doses of salicylate (Fig. 4) adapted from (Patel *et al*., 1990). Essentially, this illustrates the progressive saturation of SUA formation under conditions of increasing aspirin load to toxic amounts and raises issues about the *in vivo* glycine reserves when aspirin is taken in overdose (Patel *et al*., 1990).

If depletion of the glycine pool is of potential importance for the elimination of aspirin during overdose, then links need to be examined between glycine and HGA. One early report demonstrated in the urine of two patients, that after desalting, there were two spots observed by paper chromatography (Skarzynski *et al*., 1962). One spot had the same R_F as HGA, the other spot corresponded to a compound of HGA with glycine; after hydrolysis with acid, split into glycine and HGA lactone (Skarzynski *et al*., 1962) which has been reported elsewhere (Fiser-Herman and Petrovacki, 1958). The particularly interesting observation is that this glycine conjugate was found to represent 25% of the total amount of HGA excreted and increased in the patients' urine when oral HGA was administered (Skarzynski *et al*., 1962). It remains to be investigated whether a significant route of

HGA metabolism is through conjugation with glycine, whether hepatic glycine stores are diminished in alkaptonuria and whether supplementation of glycine would allow for enhanced excretion of a HGA-glycine conjugate.

Increasing renal elimination of HGA: Urine alkalinization is a treatment regimen that increases acidic poison elimination by the administration of intravenous sodium bicarbonate to produce urine with a pH 7.5. Most drugs at physiological pH exist partly as undissociated molecules. The extent of dissociation is a function of the ionization (acid dissociation) constant (Ka) of the drug and the pH of the medium in which it is dissolved. Ionization (dissociation) constants are expressed in the form of their negative logarithms (pKa) (Proudfoot *et al*., 2004). Hence, the stronger an acid, the lower its pKa; conversely, the stronger a base, the higher the pKa. The relationship between pKa and the proportion of total drug in ionized form is represented by the Henderson-Hasselbalch equation. When pH = pKa, the concentrations of ionized and non-ionized drug are equal. Cell membranes are more permeable to substances that are lipid soluble and in the non-ionized, rather than the ionized form (Proudfoot *et al*., 2004). The rate of diffusion from the renal tubular lumen back into the blood is decreased when a drug is maximally ionized and increased if the drug is non-ionized. As the ionization of a weak acid is increased in an alkaline environment, manipulation of the urine pH potentially can enhance renal excretion (Proudfoot *et al*., 2004). For an acidic drug, there is a greater degree of ionization at pH 8 than pH 7.4. Thus, elimination of a weak acid by the kidneys is increased in alkaline urine. Since pKa is a logarithmic function then, theoretically, a small change in urine pH could have a disproportionately larger effect on clearance, especially for those drugs that have pKa values close to blood pH. For each change in urine pH of one unit there is theoretically a 10-fold change in renal clearance whereas at best the renal clearance of a reabsorbed drug varies directly with the urine flow rate (Proudfoot *et al*., 2004). The effectiveness of urine alkalinization depends on the relative contribution of renal clearance to the total body clearance of active drug. Therefore, for HGA this should be significant. However, an aspirin overdose, even though dangerous, is a temporary 'one-off' situation. HGA formation in alkaptonuria is a lifelong over-exposure to this molecule and its metabolites. This would mean chronic, long term-to-life-time treatment for alkaptonuric patients which may itself be associated with safety issues. A potential conflicting factor in this situation is whether the extracellular fluids were to become more alkaline on sodium bicarbonate treatment. Quantitatively, the conversion of HGA to the potentially more toxic BQA would also increase in these fluids, thereby increasing the potential for BQA covalently binding to protein and the formation of reactive oxygen species, with unknown consequences for ochoronsis development. Selectively making the urine alkaline while making the tissue fluids slightly more acid would be the ideal situation for decreasing the rate of BQA formation internally and increasing the rate of HGA excretion. This might be attainable with the diuretic acetozolomide, however, this theory requires assessing in animal models.

Protein reactivity, txicity and adaptation to BQA: Similarities can be drawn between chemically reactiv drug metabolites (eg., NAPQI from paracetamol) described earlier and the oxidised form of HGA, BQA. It is electrophilic in nature and it can bind to connective tissue proteins (La Du *et al*., 1962; Zannoni *et al*., 1969), its formation or presence is associated with the production of reactive oxygen species (Martin and Batkoff, 1987). In the case of NAPQI, it is formed within the liver, due to the location of the cytochrome P450 enzymes which metabolise paracetamol to NAPQI. Also, the liver not only has a high concentration of anti-oxidants, such as GSH, at its disposal to detoxify reactive metabolites, it also has a remarkable capacity to rapidly adapt to fluctuations in oxidative stress. Hepatocytes and other cells possess oxidative stress sensors, or redox activated transcription factors, such as Nrf2 which can be swiftly mobilised to increase the production of proteins which can assist in adaptation to oxidative stress (Fig. 3) (Goldring *et al*., 2004; Park *et al*., 2005; Copple *et al*., 2008; Randle *et al*., 2008; Copple *et al*., 2010). Therefore, even though the cellular concentration of HGA and BQA in the liver is likely to be relatively high, the liver is able to defend itself against this level of oxidative stress. An important question to address would concern firstly, whether the Nrf2 or other constitutive anti-oxidant systems are involved in the detoxification of BQA. Secondly, whether these systems become saturated or less efficient as the patient ages which could lead to the emergence of the toxic manifestations observed in alkaptonuria. However, HGA can enzymatically and auto-oxidise (Martin and Batkoff, 1987). This essentially allows the HGA to travel around the body and become compartmentalised, e.g., into the synovial fluid or into areas with low blood flow, such as bone tissue, where HGA can become auto-oxidised into the protein reactive, BQA and associated reactive oxygen species (Fig. 5). BQA could covalently bind to proteins critical for cellular homeostasis, leading to cell dysfunction and cell death.

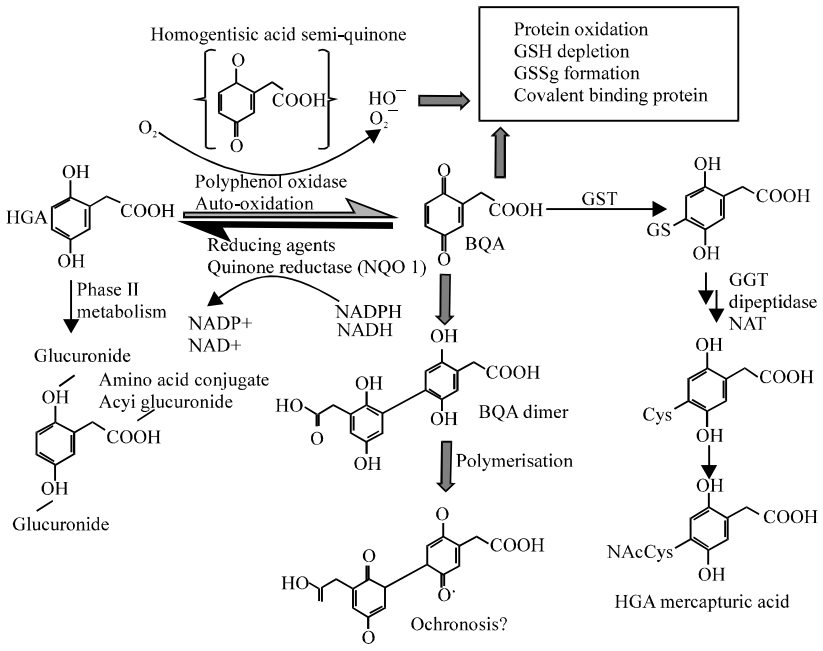

Fig. 5: Bioactivation and detoxification reactions available to HGA. HGA may undergo direct, phase II glycine conjugation. The alcohol and acid moieties in HGA are ideal substrates for glucuronidation, although this has not been reported. HGA can undergo enzyme-mediated or auto-oxidation to BQA which in turn could undergo futile redox-cycling back to HGA, with the depletion of antioxidants. BQA has the potential to dimerise with itself, potentially leading to polymerisation. BQA may become conjugated to glutathione, leading to the formation of glutathione conjugates which would be excreted in the urine in the form of mercapturic acids. BQA may covalently conjugate with protein. The production of BQA may be associated with reactive oxygen species formation (superoxide, hydroxyl radical etc). These molecules can cause protein oxidation, glutathione depletion and glutathione disulphide formation

The reactive oxygen species could also oxidise proteins leading to cell dysfunction. Indeed, in patients with ankylosing spondylitis, it has been observed that neutrophils in the circulation are primed and ready to release active oxygen species (Biasi *et al*., 1995; Ho *et al*., 2000), if these are released in body compartments with reduced detoxification capacity, this may result in adverse symptoms.

Comparison of BQA chemistry to that of similar chemical species: Clearly, when discussing the potential for BQA toxicity, we need to examine other, more widely studied quinones, one of which is benzoquinone (BQ; essentially BQA without the acetic acid side chain), a component of topical skin-lightening creams. Interconversion or redox-cycling between hydroquinone (HQ) and BQ occurs within biological matrices and results in the consumption of cellular antioxidants. Metabolism to reactive intermediates is involved in the renal toxicity associated with HQ ingestion (Fig. 6a, adapted from (Poet *et al*., 2004)). The formation of BQ is the first critical step toward the formation of toxic metabolites (Corley *et al*., 2000). While redox cycling of HQ to BQ may increase the oxidative stress associated with HQ toxicity by forming reactive oxygen species and consuming cellular reducing equivalents, the major competitive metabolic routes for HQ which are glucuronide and sulphate conjugation (Fig. 5), apparently short-circuit the redox cycling (Corley *et al*., 2000). As a result, GSH conjugation of BQ appears to play the major role in the nephrotoxicity of HQ (Monks *et al*., 1992). After the first oxidation of HQ to BQ and subsequent conjugation with GSH, the monosubstituted conjugate may be further oxidized and then conjugated repeatedly with up to four GSH equivalents (Corley *et al*., 2000). Another compound that is worth noting for its chemical similarities to HGA/BQA, is para-phenylenediamine (pPD). pPD is the most widely encountered primary intermediate in permanent hair dye formulations. Although, pPD is relatively non-toxic in most people, it is a contact sensitizer in some. On contact with skin, pPD can produce severe allergic dermatitis: a delayed cell-mediated immune

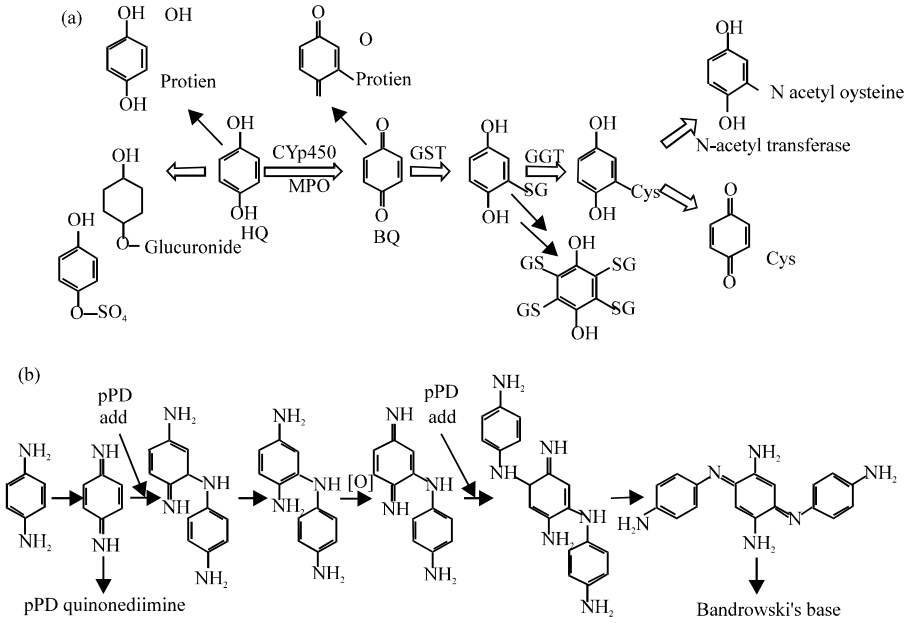

Fig. 6(a-b): Chemical similarity between the reactions of (a) Hydroquinone (HQ) and (b) *para*-phenylenediamine (pPD) with those of Homogentisic acid. (a) The chemical reactions of HQ can be used as a model to test which of these reactions HGA can undergo. Phase I oxidations, covalent binding to protein, or phase II glucuronidation, sulphation and glutathione conjugation can occur with HQ. Research is required for full metabolic profiling of HGA. (b) pPD, a major constitutent of hair dyes, undergoes oxidation to a quinonediimine which subsequently results in dimerisation of two pPD molecules. Further oxidation of the dimer, results in trimer (Bandrowski's Base) formation

response that causes skin erythema and oedema (Coulter *et al*., 2007). It is well established that for the development of contact dermatitis, pPD must provide chemical signals that activate and thereby convert dendritic cells from functionally immature antigen recognition cells into mature and potent antigen-presenting cells and act as an antigenic stimuli and thereby stimulate specific effector T-cells (Coulter *et al*., 2007; Coulter *et al*., 2008; Coulter *et al*., 2010). The effects of pPD on immune cells are thought to derive from the chemicals instability, auto-oxidation in solution results in the formation of an electrophilic quinonediimine intermediate (Fig. 6b, adapted from (Coulter *et al*., 2007)) which is susceptible to sequential self-conjugation. An end product of these oxidation-conjugation reactions is the trimer, Bandrowski's base (Coulter *et al*., 2007, 2008, 2010). There are many similarities between this type of chemical addition reaction, irrespective of the type of toxicity observed and that likely to occur with BQA and the ultimate development of ochronosis.

CONCLUSION

The formation of BQA almost certainly, from a pharmacological perspective, is the hazard initiating step, resulting in further oxidative stress, either from redox-cycling, reactive oxygen species, GSH consumption and/or protein oxidation. The multiple mechanisms and factors which are able to counteract this oxidative stress are outlined in Fig. 5. These include antioxidants (GSH), antioxidant enzymes such as those formed through Nrf2 activation (NQO1, phase II enzymes etc), metabolic pathways that are able to remove HGA, decreasing body burden, therefore lessening the capacity for forming BQA in the first instance (e.g., glycine conjugation) and finally other processes that can facilitate excretion of HGA itself (increasing renal excretion).

There is the potential for multiple toxicological mechanisms when dealing with quinone formation and redox-cycling. However, the fact remains that in alkaptonuria, the toxicological manifestations are not observed until later in life. This suggests that during the infant to teenager period, alkaptonuria patients are either not exposed to the same level of oxidative stress, or are more efficiently equipped to deal with the detoxification of the quinones or reactive oxygen species. However, it is also possible that the chemicals formed in alkaptonuria patients are not very toxic and it requires years of continual exposure to high levels of HGA before adverse symptoms appear. If this is indeed the case, more effective alkaptonuria patient lifestyle management strategies (diet, exercise etc) need to be studied for both effectiveness and

ease of patient compliance. The chronic administration of drugs reported to be effective in reducing HGA formation (e.g., nitisinone) to children from birth is not without safety issues.

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