Acyl glucuronide reactivity in perspective: biological consequences

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Abstract

The metabolic conjugation of exogenous and endogenous carboxylic acid substrates with endogenous glucuronic acid, mediated by the uridine diphosphoglucuronosyl transferase (UGT) superfamily of enzymes, leads to the formation of acyl glucuronide metabolites. Since the late 1970s, acyl glucuronides have been increasingly identified as reactive electrophilic metabolites, capable of undergoing three reactions: intramolecular rearrangement, hydrolysis, and intermolecular reactions with proteins leading to covalent drug–protein adducts. This essential dogma has been accepted for over a decade. The key question proposed by researchers, and now the pharmaceutical industry, is: does or can the covalent modification of endogenous proteins, mediated by reactive acyl glucuronide metabolites, lead to adverse drug reactions, perhaps idiosyncratic in nature? This review evaluates the evidence for acyl glucuronide-derived perturbation of homeostasis, particularly that which might result from the covalent modification of endogenous proteins and other macromolecules. Because of the availability of acyl glucuronides for test tube/in vitro experiments, there is now a substantial literature documenting their rearrangement, hydrolysis and covalent modification of proteins in vitro. It is certain from in vitro experiments that serum albumin, dipeptidyl peptidase IV, tubulin and UGTs are covalently modified by acyl glucuronides. However, these in vitro experiments have been specifically designed to amplify any interference with a biological process in order to find biological effects. The in vivo situation is not at all clear. Certainly it must be concluded that all humans taking carboxylate drugs that form reactive acyl glucuronides will form covalent drug–protein adducts, and it must also be concluded that this in itself is normally benign. However, there is enough in vivo evidence implicating acyl glucuronides, which, when backed up by in vivo circumstantial and documented in vitro evidence, supports the view that reactive acyl glucuronides may initiate toxicity/immune responses. In summary, though acyl glucuronide-derived covalent modification of endogenous macromolecules is well-defined, the work ahead needs to provide detailed links between such modification and its possible biological consequences.

Keywords: Acyl glucuronides; Reactivity; Covalent adducts; Idiosyncratic drug reactions; Drug toxicity

Abbreviations: ADR, adverse drug reaction; COX, cyclo-oxygenase; DPPIV, dipeptidyl peptidase IV; NSAID, non-steroidal anti-inflammatory drug; UGT, uridine diphosphoglucuronosyl transferase.

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1. Introduction

1.1. Focus of the review

For more than 20 years now, acyl glucuronide conjugates of carboxylate drugs have been increasingly identified and defined as reactive electrophilic metabolites. This review focuses on the biological consequences of this chemical reactivity. Apart from regeneration of the pharmacologically active drug by hydrolysis, the question of acyl glucuronide-derived toxicity (immune responses and perturbed cellular function) has arisen because of their capacity to covalently modify endogenous proteins. This review will briefly touch on the essential chemistry and biochemistry of acyl glucuronide reactivity, which are now well defined, and have been covered in several excellent earlier reviews [1–6]. The possible biological consequences of acyl glucuronide reactivity pose a key question for pharmaceutical companies: whether to continue developing carboxylate drugs when this brings with it the specter of idiosyncratic adverse drug reactions (ADRs) attributable to acyl glucuronide reactivity.

1.2. Early history of acyl glucuronide reactivity

The late 1970s flagged the first signs that all was not as had been earlier assumed with acyl (‘ester’) glucuronides, as compared to other (e.g. ‘ether’) types of glucuronides. Initially, rearrangement via acyl migration to form β-glucuronidase-resistant isomers in vitro was revealed with the acyl glucuronide of the endogenous bile pigment bilirubin, and then with the acyl glucuronide of the antilipidemic drug clofibric acid [7–9]. Then followed reports of rearrangement in vitro of the acyl glucuronides of a multitude of drugs, continuing to this day. Also in the late 1970s, it was reported that renal failure patients taking carboxylate drugs showed decreased drug clearance, and this was suggested as systemic hydrolysis leading to conjugation–deconjugation (‘futile’) cycling [10,11]. The possibility of hydrolysis of acyl glucuronides in biological samples ex vivo was also soon realized [12]. In the early 1980s, the potential for bilirubin to become covalently attached to serum albumin (‘biliprotein’) in hepatobiliary/cholestatic disease was recognized as being attributable to bilirubin acyl glucuronide [13,14]. At about the same time, in the first review of acyl glucuronide reactivity, Faed [1] predicted that drug acyl glucuronides could covalently interact with endogenous proteins, with possible toxic consequences. Landmark papers describing such covalent interactions appeared in the mid 1980s [15–17]. Since then, there has followed a multitude of in vitro reports documenting acyl glucuronide–derived drug–protein adducts, pointing to possible immune/toxic sequelae in vivo. Reports of formation of drug–protein adducts in vivo (presumably acyl glucuronide–derived) have also appeared, and continue. It is against this background that the current review seeks to place the question of biological consequences in perspective.

2. Reactivity of acyl glucuronides—chemical/biochemical aspects

2.1. Intramolecular rearrangement

The essential organic chemistry underpinning pH-dependent acyl migration between adjacent hydroxy groups on polyhydroxy compounds has been known since the early 20th century [18,19]. After some 20 years of research by drug metabolism workers, this phenomenon is now orthodoxy for acyl glucuronide metabolites of carboxylate compounds. Thus, the biosynthetic 1-O-β-acyl glucuronide can undergo pH-dependent (OH−catalyzed, occurring at neutral to slightly alkaline pH) migration of the drug moiety to the 2-O-, 3-O- and 4-O-positions (Fig. 1). These migrations are readily reversible, except that reformation of the biosynthetic, higher energy 1-O-β-acyl glucuronide is highly unfavorable. Recent work, however, does document that reversible 2-O- to 1-O-acyl migrations occur in both the β- and α-configurations [20,21].

Nomenclature is important here. As pointed out by Janssen et al. [22], the term ‘glucuronide’ should be reserved for the product of biosynthesis by uridine diphosphoglucuronosyl transferases (UGTs) (the 1-O-acyl-β configuration, susceptible
to β-glucuronidase hydrolysis). Acyl migration rearrangement isomers are non-biosynthetic (they are the products of chemical rearrangement of the glucuronide), are resistant to β-glucuronidases and should be referred to as ‘acyl-substituted glucuronic acids’ or preferably ‘isomers of the acyl glucuronide’.

The 2-O-, 3-O- and 4-O-β acyl migration rearrangement isomers, unlike the biosynthetic 1-O-β-acyl glucuronide itself, can ring-open with transient formation of a planar aldehyde group at C1. Subsequent cyclization leads to reformation of the 1-OH-β or formation of the 1-OH-α configuration at C1 (i.e. anomerization or ‘mutarotation’, Fig. 1). The biosynthetic 1-O-acyl-β glucuronide is much more susceptible to nucleophilic substitution reactions (because of the proximity of the ring oxygen atom) than its acyl migration isomers. However, the 2-O-, 3-O- and 4-O-acyl migration isomers are potentially reactive to form imines via the aldehyde group of their transient ring-opened forms (which are much more reactive than their ring-closed β- or α-anomers) (this becomes important in mechanisms of covalent adduct formation, see Section 2.3 below).

Thus, two processes occur during acyl glucuronide rearrangement: acyl migration (reversible migrations amongst neighboring OH groups), and anomerization (reversible conversion from β- to α-configuration at C1).

### 2.2. Hydrolysis

Whereas an acyl glucuronide can be susceptible to hydrolysis by a myriad of catalysts, including β-glucuronidases (by definition), non-specific esterases, serum albumin and hydroxide ion, the same catalysts can hydrolyze the acyl migration isomers, with the notable exception of β-glucuronidases. The capacity of acyl glucuronides to be hydrolyzed in a comparatively facile way contrasts with the relative intransigence to hydrolysis of corresponding ether/phenolic glucuronides (‘ester’ groups are much more chemically fragile than ‘ether’ groups). It might be expected that ether/phenolic glucuronides will withstand hydrolysis under conditions in vitro and in vivo where acyl glucuronides might be readily cleaved.
2.3. Intermolecular covalent modification of proteins

Faed [1] predicted in 1984 that acyl glucuronides would covalently modify endogenous proteins, because of their capacity as electrophiles to cause substitution reactions with nucleophilic groups located on proteins or other macromolecules, and that this could ultimately lead to toxic consequences. This prediction was based upon the ‘transacylation’ mechanism (Fig. 1), subsequently promulgated by van Breemen and Fenselau [15] and Ruelius et al. [16] in their reports of drug acyl glucuronide-derived covalent modification of serum albumin. The alternative ‘Schiff’s base’, ‘imine’ or ‘glycation’ mechanism (Fig. 1) was initially proposed by Smith et al. [17,23]. It is analogous to the non-enzymic glycation of albumin by glucose and other sugars in vivo. Though it requires prior acyl migration of the glucuronide, this mechanism can contribute more to the covalent adduct yield than the transacylation mechanism [24–28]. Adduct stability is altogether another question [29]. Ruelius et al. [16] noted that transacylation with nucleophilic sulfydryl (mercapto), hydroxy or amino groups located on protein residues would lead to thioester, ester and amide linkages (respectively) between the drug and protein moieties, and that these would have considerably different chemical stabilities (thioester < ester < amide). On the other hand, the glycation mechanism involves the reversible condensation of the reactive aldehyde group (transiently formed during anomerization of the 2-, 3- and 4-isomers) and a primary amino group on protein (Fig. 1). Theoretically, the imine so formed could undergo Amadori rearrangement to a more chemically stable amino–ketone linkage (analogous to structures originating from glycation of albumin by glucose [30]), but this (or other possible end structures) has not yet been formally documented in the case of drug acyl glucuronides. It is worth noting that the ‘glycation’ mechanism leads to structures in which the glucuronic acid residue is retained in the adduct (drug still bound to glucuronic acid [via an ester linkage] which is, in turn, bound to protein, Fig. 1). This contrasts with the transacylation mechanism, in which the glucuronic acid residue is lost from the covalent drug–protein adduct.

2.4. Comparison of acyl glucuronide reactivity

All acyl glucuronides have a major common element of chemical structure (i.e. the 1-O-acyl-β-D-glucuronic acid moiety, Fig. 1). However, acyl glucuronide reactivity can differ enormously, and this obviously depends upon the electronic, steric and other characteristics of the drug (‘R’ moiety in Fig. 1). (In this context, it is worth noting that no formal systematic structure–reactivity relationship has been conducted to date.) Perhaps the most useful comparative measure of intrinsic chemical reactivity of an acyl glucuronide is the half-life of degradation in a protein-free environment (proteins can accelerate, retard or have no effect upon acyl glucuronide degradation). Thus, in a protein-free buffer at pH 7.4 and 37 °C, half-lives of degradation (mainly rearrangement under these conditions) range from about 15 to 30 min for isoxepac [31], zomepirac [32], tolmetin [33] and probenecid [34] to about 3 days for valproic acid [35].

3. Reactivity of acyl glucuronides—biological aspects

3.1. Intramolecular rearrangement

The isomerization of acyl glucuronides by intramolecular rearrangement (either acyl migration or anomerization) is catalyzed by hydroxide ion (although the presence of albumin can have a modulating effect). The event per se should have no biological consequences. However, it does have major ramifications (Fig. 2). The biosynthetic acyl glucuronide can be more readily hydrolyzed (cleaved by β-glucuronidases and other catalysts), and will more readily engage in transacylation reactions (with nucleophilic groups on proteins and other macromolecules) as compared to its acyl migration rearrangement isomers. The rearrangement intermediates are less readily available to hydrolysis, but can be better proponents of covalent
adduct generation (via the glycation mechanism) than the acyl glucuronide itself [27,35].

3.2. Hydrolysis

3.2.1. Enterohepatic recirculation

Enterohepatic recirculation (biliary excretion of acyl glucuronide, hydrolysis in the gut, reabsorption of parent drug into the portal blood stream, reuptake by the liver) is a well-established phenomenon, and will be touched upon only briefly. Most of the in vivo work documenting this conjugation–deconjugation cycle comes from animal (particularly rat) studies. A useful example is the comparative dispositions of valproic acid, its acyl glucuronide and rearrangement isomers in the rat. Some 40–60% of valproic acid doses are excreted in the bile of rats as the acyl glucuronide conjugate [36]. Enterohepatic recirculation (abolished by bile exteriorization) causes secondary increases in valproic acid blood concentrations of some two- to threefold. Similar situations must be envisaged for humans, though the molecular weight/size ‘threshold’ for significant biliary excretion for organic anions, now known to be mediated primarily by the canalicular MRP2 transporter [37], is greater in humans (∼500–600 Da) than in rats (∼300–400 Da) [38,39]. In contrast to the ready hydrolysis of the acyl glucuronide of valproic acid in the gut of the rat, the acyl glucuronide isomers were much more resistant to hydrolysis [40]. The same relative resistance to hydrolysis in the gut was found for the isomers of the acyl glucuronide of diflunisal, as compared to the acyl glucuronide itself [41]. With this particular drug, it was also possible to show that the phenolic glucuronide was more resistant to gut hydrolysis than the acyl glucuronide [41]. Thus, the portion of a drug acyl glucuronide excreted in bile that manages (dependent on
intrinsic reactivity) to rearrange in the time between canicular excretion and passage into the small intestine (and consequent exposure to β-glucuronidases and esterases) has a much greater chance to reach the large intestine [41]. The obvious biological consequences of enterohepatic recirculation are to increase the residence time of the drug in the body [41]. Apart from enterohepatic cycling, biliary excretion of acyl glucuronides may also have significant consequences resulting from covalent drug–protein adduct formation in the gut (see Section 3.3.6.3).

3.2.2. Systemic hydrolysis

Recognition of systemic conjugation–deconjugation cycling of acyl glucuronides post-dated recognition of enterohepatic conjugation–deconjugation cycling. As noted earlier (Section 1.2), evidence that acyl glucuronides were susceptible to systemic hydrolysis initially came from studies in human renal failure patients, where it was shown that renal insufficiency (with compromised capacity to excrete an acyl glucuronide in urine) was associated with reduced plasma clearance of the parent drug [10,11]. There is now no doubt that dosage of carboxylate drugs forming acyl glucuronides as significant metabolites needs to be carefully monitored in patients with compromised renal function.

Studies in animals, particularly rats, have been instructive here. One might have thought that enhanced systemic exposure of the acyl glucuronide to systemic β-glucuronidases, as a result of renal insufficiency, would be the primary reason for the reduced drug clearance. Studies with clofibric acid in rabbits [42,43] and zomepirac in guinea pigs [44] have clearly shown that esterases, rather than β-glucuronidases, are the primary hydrolytic catalysts for acyl glucuronides in these circumstances. In rabbits with impaired renal function (caused by uranyl acetate administration), plasma clearance of diphenylacetic acid was decreased by 35%; renal clearance of the acyl glucuronide was decreased by 70% [45]. This renal impairment had little effect on hydrolysis clearance of the acyl glucuronide, but significantly reduced its formation clearance. As noted earlier (Section 2.4), acyl glucuronides degrade in the test tube primarily by rearrangement. However, hydrolysis plays the dominant role in vivo. This is exemplified by comparison of the plasma profiles obtained after (separate) i.v. administration of diflunisal, its acyl glucuronide, isomers of the acyl glucuronide and the phenolic glucuronide in rats [46]. Less than 10 min after i.v. injection of (β-glucuronidase-susceptible) diflunisal acyl glucuronide into rats, the plasma concentrations of the parent drug exceeded those of its acyl glucuronide. Systemic hydrolysis of the (β-glucuronidase-resistant) isomers of the acyl glucuronide was much slower, and none was observed after i.v. administration of the (β-glucuronidase-susceptible) phenolic glucuronide (further attesting to the possible irrelevance of β-glucuronidases in the systemic hydrolysis of glucuronides).

Apart from reduced clearance of pharmacologically-active parent drug (caused by systemic hydrolysis of acyl glucuronides and exacerbated by renal insufficiency), systemic conjugation–deconjugation cycling can have other interesting biological consequences. This is elegantly illustrated in the recent work by Grubb et al. [47], using the chiral arylopropionic acid non-steroidal anti-inflammatory drug (NSAID) ketoprofen. In renal failure patients administered ketoprofen (as the racemate, a 50:50 mixture of the R- and S-enantiomers), compromised capacity to excrete R- and S-ketoprofen acyl glucuronide in urine resulted in enhanced regeneration of parent drug by hydrolysis. In turn, because of the underlying (acyl CoA-driven) chiral inversion of (pharmacologically-inactive) R-ketoprofen to (pharmacologically-active) S-ketoprofen, this led to ‘a disproportionate increase in systemic exposure to the S-enantiomer that inhibits both pathologic and homeostatic prostaglandin synthesis’.

We wish to make a final comment on terms used to describe conjugation–deconjugation cycling. The term ‘futile cycling’, proposed 20 years ago to describe systemic conjugation–deconjugation cycling of carboxylate drugs, has gained some acceptance in the literature. We contend that this term is inappropriate, as it places a value judgment on, rather than a description of, the process. ‘Futile cycling’ was coined at a time when no-one suspected rearrangement and covalent binding
3.3. Intermolecular covalent modification of proteins

3.3.1. Overview of adduct formation

The covalent binding of carboxylate drugs, via their acyl glucuronide metabolites, to proteins is now a very well-established phenomenon in vitro. Under well-defined conditions, the extent of covalent binding of an acyl glucuronide-forming drug to serum albumin in vitro is somewhat predictable from the acyl glucuronide degradation rate ($t_{1/2}$ for intramolecular rearrangement and hydrolysis in buffer at 37 °C at pH 7.4, Section 2.4) [48]. That is, the (initial) rate of covalent binding to serum albumin is directly related to reactivity and concentration of the acyl glucuronide in the buffer. In this context, it is worth noting that, although acyl glucuronides are chemically reactive electrophilic metabolites, they are, as a class, relatively stable compared to many other classes of reactive metabolites which do not survive beyond the immediate environment in which they were produced. By contrast, acyl glucuronides can (at least in part) escape the cell, circulate around the body, and be excreted in urine or bile. Thus, the spectrum and potentially wide distribution of their covalent interactions with tissue macromolecules makes identification of critical targets and any biological consequences very difficult.

Although absolute proof of the intermediary of acyl glucuronides in covalent drug–protein adduct formation in vivo is lacking, there is enough direct evidence (glucuronidation inhibitors decrease extent of covalent binding of diclofenac and zomepirac in hepatocytes in culture or suspension [49,50]) and circumstantial evidence which, when coupled with the extensive in vitro documentation of acyl glucuronide-derived protein modification, makes the case probable. However, it should not be forgotten that carboxylate drugs can also be substrates for acyl CoA ligases, thereby forming reactive acyl CoA thioesters that may be capable of forming covalent drug protein adducts in vivo [51–59]. Indeed, recent work on a model arylpropionic acid substrate compared the potential importance of acyl CoA-derived versus acyl glucuronide-derived covalent modification of macromolecules [60]. Quite possibly, both acyl glucuronides and acyl CoAs contribute to the overall drug–protein adduct load (at least in liver). On the other hand, it has been suggested that diclofenac-derived protein adducts were generated following metabolism by cytochrome P450 and UGT, but not by acyl CoA ligase action [61]. In spite of the possibility of acyl CoA-derived adducts in liver, the in vivo covalent binding of carboxylate drugs to plasma proteins of humans and rats may be somewhat predictable (at least before steady state or equilibrium has been established with chronic dosing) if the extent of adduct formation is correlated with the extent of exposure measured by area under the plasma acyl glucuronide concentration–time curve [48,53]. Overall, it is safe to conclude that covalent drug–protein adducts will form in many tissues of human patients taking acyl glucuronide-forming carboxylate drugs therapeutically.

3.3.2. Adverse reactions to carboxylate drugs

Some of the most commonly prescribed and over-the-counter drugs are carboxylate compounds, including many NSAIDs and the fibrate lipid lowering drugs. Around 25% of drugs withdrawn from marketplaces around the world due to severe toxicity have been carboxylic acids [62–64]. These include the NSAIDs alclofenac, ibufenac, bendazac, zomepirac, bromfenac, fenofenac, pirprofen, indoprofen, suprofen, benoxaprofen and flunoxaprofen. Zomepirac was withdrawn (March 4, 1983) relatively soon after release (November 1980) after a number of unexplained fatal anaphy-
lactic reactions (see [65]). It is worth mentioning the anti-epileptic agent valproic acid in this context. Although valproic acid has been associated with patient deaths due to serious idiosyncratic hepatotoxicity (132 fatalities were traced worldwide from 1979 to 1994 [66]), it is generally considered a safe drug, and continues to enjoy increasing market share.

3.3.3. Mechanisms of ADRs

ADRs can be classified as intrinsic (which are unique to the drug and may occur in all people if exposed to high enough concentrations) or idiosyncratic (not predictable and, until recently, not thought to be dose related). The range of idiosyncratic ADRs attributed to acyl glucuronide forming drugs is large and diverse, and very occasionally life threatening. For NSAIDs, idiosyncratic ADRs have been demonstrated affecting many tissues including those of hepatic (hepatotoxicity is considered a class characteristic of NSAIDs), metabolic, endocrine, cardiovascular, dermatological, gastrointestinal, genitourinary, renal, hematological, musculoskeletal, and neurological origin (see Ref. [67]). The most serious ADRs to NSAIDs have usually occurred in liver and kidney, organs associated with high exposure to acyl glucuronides. Potentially fatal ADRs most often seem to be immunologically based. These include anaphylactic reactions and severe dermatological reactions such as Stevens–Johnson syndrome and fatal epidermal necrolysis. Currently, the precise mechanisms underlying NSAID-induced hepatotoxicity (allergic hepatitis, hepatobiliary disease), immunocytopenias (leukopenia, thrombocytopenia, eosinophilia, agranulocytosis, neutropenia, hemolytic anemia) as well as hypersensitivity reactions (dermatological, multisystem, hepatic) are unknown. The presence of rashes, fever, and/or eosinophilia in some patients, which are indicative of hypersensitivity reactions, does suggest the immune system may be involved. However, these markers are absent in other patients, raising the possibility that direct toxicities caused by the parent drugs and/or their metabolites could be responsible. A common mechanism that might underlie idiosyncratic toxicity is the acyl glucuronide-derived formation of NSAID-modified proteins.

Can a link between covalent modification of proteins and idiosyncratic ADRs be demonstrated experimentally? If covalent binding was a cause of these ADRs, two mechanisms could be involved.

– immune idiosyncrasy, where modification causes a self protein to somehow become a target of the immune system; in these cases an antibody (or T-cell) could be directed at just the drug or a combination of drug and protein, or the modification may allow the generation of an antibody directed solely to the protein (e.g. the anti-liver/kidney microsome {anti-LKM} autoantibodies found in patients with immunooallergic drug-induced hepatitis [68]);

– functional idiosyncrasy, where the modification of an amino acid residue at or near an important part of the protein (e.g. the active site of an enzyme or binding site of a protein) causes the malfunctioning of that protein.

Indeed, because of the diversity of ADR seen with NSAIDs, both these mechanisms may be needed to explain different side effects, and some evidence for both these mechanisms has appeared in the literature.

3.3.4. Possible mechanisms of immune mediated injury

The long-standing Gell and Coombs’s classification divides drug allergies into four types—allaphylaxis (Type I), antibody-mediated cytotoxic reactions (Type II), immune complex-mediated reactions (Type III), and delayed type hypersensitivity (Type IV) [69]. This classification has been challenged as outdated [70]. These authors suggest it be replaced by a scheme with three divisions: (i) antibody mediated; (ii) cell mediated; and (iii) pseudoallergic reactions (e.g. due to inflammatory mediators such as cytokines). If the possibility of two or all three mechanisms working in concert is taken into account, this system should cover all drug hypersensitivity injury.

3.3.4.1. Immune sensitization. The initial events eliciting immune sensitization (antibody produc-
tion, T-cell sensitization or production of immune mediators such as histamine or cytokines) in response to drug therapy have not been verified, and antibody production often occurs in patients who do not later experience pathology. In fact, adverse events involving immune reactions are uncommon (although they make up a large proportion of ADR when they do occur), and exposure to genetic and environmental factors (or age) may be necessary before toxicity develops. Competent metabolic and immune systems are probably also required for an immune based toxicity (see Section 3.3.7 on individual susceptibility).

Orthodox immunology: For an immune response to be elicited by a drug, it is generally thought that it must first become covalently bound to a carrier protein (i.e. the ‘hapten hypothesis’). As most drugs are chemically unreactive, metabolism to a reactive intermediate that can covalently interact with a protein must first occur [71]. However, there is evidence that close association with cell surface proteins on (e.g.) blood cells may be enough to stimulate the immune system [72,73].

Danger hypothesis: A new paradigm is gaining some acceptance in immunology. The ‘danger hypothesis’ of immune sensitization suggests that components, released or induced during cell or tissue injury, are required to stimulate the immune system [74–76]. Foreignness (or non-self) of an antigen alone is insufficient to stimulate an immune response and in some cases is not needed (e.g. autoimmune responses). For example, if there is tissue damage, local antigen presenting cells (e.g. dendritic cells) take up local antigens (both drug modified proteins and normal self proteins) and so-called danger signals stimulate them to migrate to the lymph nodes and present antigen, and stimulate T- and B-cell immunity. If the dendritic cells receive no danger signals, rather than inducing immune responses, they induce tolerance. Covalent adduct formation could be envisaged to damage cells (e.g. hepatocytes) thus providing the danger signal (stimulating the dendritic cells). Liver enzyme levels in the plasma are often elevated during NSAID use, suggesting leakage of intracellular contents. These could supply the danger signal necessary in this scheme. With continued dosing, this damage may continue, maintaining stimulation of immune responses against drug-modified cell components, and/or an autoimmune reaction against normal self-proteins. If dosing of the drug is discontinued, the ‘danger signals’ are likely to cease, immune activation curtailed and tolerance induction returned, resulting in abatement of both anti-drug adduct and auto-immune responses. This hypothesis could explain many things about NSAID immune reactions, such as autoantibody production and the fact that drug allergies almost invariably cease soon after the drug is discontinued, despite the fact that drug modified proteins persist in vivo for more than a week [77]. This theory could prove a useful probe to further evaluate immune-based adverse reactions.

3.3.5. Evidence for involvement of acyl glucuronides in generating an immune response

3.3.5.1. Drug specific antibodies or sensitized T cells. In 1992, it was reported that nine of 57 patients being chronically treated with the antiepileptic agent valproic acid showed measurable levels of antibodies to valproate–albumin adducts (obtained by incubating isomers of valproic acid acyl glucuronide with human serum albumin) [35]. However, the titres were very low and clinically insignificant. While this demonstrates an immune sensitization, it has not been linked to immune damage. Although valproic acid (which forms a very stable acyl glucuronide: $t_{1/2}$ $\sim$ 3 days, Section 2.4) has caused fatalities due to idiosyncratic hepatotoxicity, it does not seem to do so by immunological processes, and its acyl glucuronide is considered too unreactive to be the causative agent for these reactions [35].

Two papers in 1995 provided the first experimental evidence for a possible immune response to acyl glucuronide-derived drug–protein adduct formation with the demonstration of drug-specific antibodies generated in rats and mice following immunization with rat or mouse serum albumin (respectively) that had been covalently modified by diflunisal or tolmetin acyl glucuronides (respec-
tively) [78,79]. As these immunizations were all carried out with antigen prepared in vitro (with epitope density possibly much greater than what might be expected in vivo) in the presence of adjuvant (which heightens the immune response and may provide a 'danger signal'), they therefore do not directly answer the question: can or do natural (in vivo-derived) acyl glucuronide-generated drug–protein adducts sensitize the immune system?

In an interesting experiment, published in 1995 by Boelsterli’s group [80], splenocytes from mice immunized with diclofenac-keyhole limpet hemocyanin (and exhibiting high anti-diclofenac antibody titers) were isolated and co-cultured with syngeneic murine hepatocytes pre-exposed to diclofenac. This led to increased liver enzyme levels in the culture media (a marker for hepatocyte injury and possibly a ‘danger signal’ in vivo) when compared to either non-sensitized lymphocytes or control hepatocytes. In this case the hepatocytes would be expected to contain diclofenac adducts (as has been demonstrated for zomepirac [50]), most of which would be membrane bound. That is, for cellular (hepatocyte) injury, the lymphocytes had to be sensitized to diclofenac and the hepatocytes exposed to this drug i.e. the sensitized lymphocytes recognized diclofenac-modified proteins on the hepatocytes and the result was immune-mediated damage. The extent of cell injury was highest in the presence of lymphocytes highly enriched in T cells, and was much reduced in the presence of anti-MHC class I antibodies (suggesting MHC I-dependent recognition). The addition of co-culture supernatants to hepatocytes had no effect, thus ruling out the possibility that soluble factors alone mediated the cell injury. Evidence was also presented for an antibody-dependent cell-mediated mechanism of injury. Non-stimulated lymphocytes in the presence of soluble factors (antibodies) from the culture supernatant of diclofenac-stimulated lymphocytes could also damage these hepatocytes. Although these results initially sound very convincing (as far as demonstrating an immune-based mechanism), it must be remembered that it relies on the artificial stimulation of the immune system by direct immunization with an artificially modified drug–protein adduct in the presence of adjuvant. If anything (as hepatocytes cultured with an acyl glucuronide forming NSAID will lead to drug covalent binding to hepatocytes [49,50]), it shows how efficient the immunization process is, stimulating both a T- and B-cell response.

3.3.5.2. Drug-induced immunocytopenias (blood dyscrasias). Some of the most striking seemingly immune-derived ADRs are immunocytopenias. These include disorders of several blood cell types (leukopenia, thrombocytopenia, eosinophilia), agranulocytosis, neutropenia, hemolytic anemia). Although these reactions are rare, they are almost universal i.e. drugs from many classes have been demonstrated to cause these pathologies [81]. Zomepirac, ibuprofen, suprofen, sulindac, fenoprofen, tolmetin, etodolac, mefenamic acid, diclofenac, diflunisal, naproxen and aspirin (as well as other non-carboxylate NSAIDs such as feprazone) [81] have all been linked to immunocytopenias. Immunocytopenias seem to occur by perhaps three [82] or four [83] mechanisms. Some authors suggest that all can be explained by a single mechanism [84]. This latter contention specifies that a drug or a drug metabolite interacts with (e.g.) a cell membrane protein(s) generating epitopes that are recognized by the immune system. This elicits production of antibodies that may react with drug or metabolite, a mixture of drug or metabolite and membrane protein, or membrane protein alone.

In 1993, in a case of ibuprofen-precipitated thrombocytopenia, it was shown that IgM and IgG antibodies in the patient’s blood were able to bind to platelets in the presence of urine from a volunteer taking ibuprofen [85]. Less binding was observed with the drug itself. This implies that a metabolite was necessary for the antibodies to recognize the platelets and suggests a possible role in immune sensitization.

In 1996, it was demonstrated that all 17 of 17 patients with diclofenac-induced immune hemolytic anemia had antibodies that reacted with erythrocytes in the presence of ex vivo antigen (urine containing drug and metabolites {including acyl glucuronides}) or drug. Four of these patients showed reactions only with urine [71]. This sug-
gests that these four reacted solely with the metabolite portion of the urine, which for diclofenac would be mostly the acyl glucuronide (and/or its rearrangement isomers).

In 1997, remarkable work was published showing that a patient had antibodies to the acyl glucuronide of 4'-hydroxy diclofenac (a phase I metabolite of diclofenac) [86]. This patient presented with acute hemolytic anemia, and was found to have an IgM antibody that reacted strongly with erythrocytes in the presence of urine from patients taking diclofenac. While NSAIDs strongly interact with erythrocytes in the presence of urine glucuronide of 4'-hydroxy diclofenac (a phase I metabolite of diclofenac) [86]. This patient presented with acute hemolytic anemia, and was found to have an IgM antibody that reacted strongly with erythrocytes in the presence of urine from patients taking diclofenac. While NSAIDs have been implicated in immunocytopenias before (hundreds of papers implicating NSAIDs have appeared since the late 1960s) and there was evidence of antibodies to drug (e.g. tolmetin [87]) and to drug and metabolites (e.g. sulindac and its sulfone and sulfide metabolites [88]), this was the first time an acyl glucuronide metabolite had been directly implicated.

More evidence for metabolite-specific antibodies appeared in 1997. A patient with acute hemolytic anemia was shown to have diclofenac-dependent antibodies that reacted with erythrocytes only in the presence of urine from a volunteer receiving diclofenac [89]. This again suggested the likelihood of a metabolite(s) (diclofenac acyl glucuronide?) being the antigen (though it should be remembered in this context that diclofenac does form a reactive P450-derived metabolite). On only two other occasions have drugs (etodolac and naproxen) been shown to elicit antibodies that depend on acyl glucuronides [82,90] (though this may be because analysis of such compounds in this context has rarely been performed). The involvement of glucuronide metabolites was again suggested. Like diclofenac, both the acyl glucuronide of a phase I metabolite (i.e. 6-OH etodolac acyl glucuronide) and the acyl glucuronide of etodolac itself were implicated. The antibodies to the etodolac glucuronides were reactive against erythrocytes. The antibodies to naproxen glucuronide were active against platelets. The fact that these antibodies were sometimes reactive with metabolite but not drug reinforces the possibility of a covalent adduct derived from an acyl glucuronide being responsible for the sensitization.

The abundance of material linking immune disorders of blood cells and the use of NSAIDs certainly suggests a causative role for these compounds. The requirement of acyl glucuronide metabolites as antigens in some cases is strongly suggestive of an immune sensitization taking place. However, no unequivocal direct involvement of an acyl glucuronide derived drug–protein adduct has been demonstrated. Indeed some NSAIDs that apparently trigger immunocytopenias (e.g. feprazone, azopropazone, phenylbutazone and piroxicam [81,91–93]) are not carboxylic acids. This leaves open the possibility of a pharmacological mechanism being the trigger. Is it possible that the only thing different for acyl glucuronide-forming drugs and other drugs implicated in immunocytopenia initiation is the mechanism of becoming covalently or closely associated with cellular proteins?

3.3.5.3. Mycophenolic acid. Mycophenolate mofetil is an immunosuppressant prodrug used in gram quantities with solid organ transplants. The active agent, mycophenolic acid, is metabolized in the liver primarily to a phenolic glucuronide. An acyl glucuronide is also formed. This metabolite was suggested to be as pharmacologically active as mycophenolic acid, demonstrating a similar capability to inhibit lymphocyte proliferation in vitro [94], but these results could have been explained by a high level of hydrolysis to reform the parent drug. Nonetheless, this acyl glucuronide metabolite was shown to specifically induce cytokine (TNFα and IL-6) formation in leukocytes in culture (mycophenolic acid and its phenolic glucuronide were inactive [glucuronic acid was not tested]) [95], and therefore appears to have both immunosuppressive and pro-inflammatory activity in vitro. It could be envisaged that induction of immune modulators could somehow lead to immune ADR. Interestingly, prostaglandins seem to be potent inhibitors of TNF release from macrophages [96], and therefore a link can be made with NSAIDs which inhibit prostaglandin synthesis and therefore may up-regulate production of specific cytokines. Recently, it has been demonstrated that mycophenolic acid acyl glucuronide–albumin adducts were formed in all patients after short- or
long term dosing [97]. The similarities between mycophenolic acid and NSAIDs in chemical shape, metabolism, side effect profile [98,99] and general use as anti-inflammatory agents suggests similar mechanisms could be at play.

3.3.5.4. Oral tolerance to NSAIDs. The mechanisms of oral tolerance are unknown. However, a requirement for induction and maintenance of tolerance is the interaction of soluble antigens with gut-associated lymphoid tissue. The work of Ware et al. with diclofenac [100] suggested that acyl glucuronide-derived intestinal protein adduct formation may lead to the down-regulation of drug-induced allergic reactions, and this may be the reason why immune reactions are relatively uncommon.

3.3.6. Possible mechanisms of metabolic or functional idiosyncratic ADRs

3.3.6.1. Dipeptidyl peptidase IV. In 1995, a 110 kDa band from diclofenac dosed rats (this band has subsequently been shown to be modified in the liver of rats and mice dosed with zomepirac [53], diclofenac [61], diflunisal [53], sulindac [101] and ibuprofen [101]) was identified to be at least partly made up of the membrane enzyme dipeptidyl peptidase IV (DPPIV, also known as CD26) [102]. Its activity was also shown to be decreased in plasma membrane fractions from livers of these animals. Apart from serum albumin, this was the first of modified proteins to be identified. Interestingly, soluble CD26 attains significant levels in serum, and has been shown to influence T-cell proliferation [103,104]. DPPIV has been demonstrated to be localized to the apical (bile canaliculär) membrane in hepatocytes [105], and could well become a target during biliary excretion of acyl glucuronides through this membrane. Recent work shows convincingly that DPPIV is not the only protein modified at a MW of ~110 kDa, as even in DPPIV-deficient mice, there is still a band at 110 kDa detected by anti-zomepirac antibodies after zomepirac dosing [106]. These results confirm that DPPIV is modified by zomepirac (and therefore most likely by other drugs forming reactive acyl glucuronide metabolites undergoing biliary excretion), but that it constitutes only a portion of the protein modified at this molecular weight. Researchers initially targeted this band as it was one of the major modified-protein bands identified by western blotting.

3.3.6.2. UGTs as targets of acyl glucuronide metabolites. In 1999, ketoprofen acyl glucuronide was shown to covalently modify human and rat liver UGTs in vitro [107]. The covalent binding of ketoprofen, mediated by ketoprofen acyl glucuronide, to human hepatic microsomes and to membrane fractions from UGT2B1-transfected V79 cells also caused concentration-dependent inhibition of glucuronidation of naphthol in these samples. This inhibition was irreversible and associated with an increase in the amount of total protein modified, suggesting that modification of proteins, including UGTs, led to inactivation of the modified enzyme. This raises the question of whether reactive acyl glucuronide metabolites could covalently interact with and inhibit the UGT enzymes which formed them in vivo. However, in other work where rats were dosed daily for 35 days with high (50 mg/kg) doses of the NSAID diflunisal (that forms a reactive acyl glucuronide as its major metabolite in rats), there was no evidence for compromised biosynthesis of glucuronides or hepatobiliary function [108]. This argues against the notion of acyl glucuronide inactivation of UGTs in vivo. Autoantibodies to UGTs have been described in patients with autoimmune or viral hepatitis [109]. Therefore, NSAID–UGT adducts could possibly be acting as autoantigens in humans. While similar modification of UGTs in vivo is likely, whether the formation of such adducts has any biological implications is not evident from these experiments.

3.3.6.3. Ulceration of small intestine. Another unexplained NSAID injury is ulceration of the small intestine. Up to 70% of patients on chronic NSAID therapy develop small intestinal enteropathy that includes chronic blood and protein loss. Small intestinal injury has received more attention after it became clear that it is not closely associated with the familiar gastric ulceration seen with NSAIDs. In particular, there is evidence that
prostaglandin synthesis inhibition may not be directly involved [110,111]. Several alternate mechanisms have been proposed for small intestinal injury. These mechanisms include roles for changes in mitochondrial energy production, enterobacteria, bile, oxygen radicals, toxic mediators from neutrophils and enterohpatic circulation. Of these, the presence of bile and bacterial flora and an intact enterohpatic circulation all seem to influence the enteropathy and play important roles in the severity of ulceration.

NSAIDs could reach the small intestine after oral ingestion or (as metabolites) via biliary excretion. The importance of an intact enterohpatic circulation in small intestinal ulceration was emphasized by Boelsterli [112] and demonstrated by Atchison et al. after a single dose of diclofenac [113]. In this study, a single dose of diclofenac was shown to lead to dose-dependent formation of drug–protein adducts and small intestinal ulcers only in animals with intact enterohpatic circulation. Adducts were formed within an hour of dosing and their formation preceded ulceration and protein leakage from the blood circulation. Furthermore, the areas of most drug–protein adduct formation were found to be coincident with those with most ulceration. (Indeed the most protein adducts in rats dosed with diflunisal were found (in order) in the liver, kidney, plasma and small intestine [114].) Neither adducts nor ulcers were found in animals with bile drainage or bile duct ligation. These results taken as a whole suggest a possible causal role for drug–protein adducts in ulceration. Other studies have reinforced these results. Boelsterli and coworkers [115,116] have shown that transport-deficient mutant rats (which lack the mrp2 exporter and are unable to secrete diclofenac-glucuronides into bile) were refractory for diclofenac-induced small intestinal ulceration. These animals developed more severe ulcers after oral administration of bile containing diclofenac-glucuronide compared to animals receiving normal bile mixed with free diclofenac. Interestingly, the mrp2 transporter protein appeared not to be a target for covalent modification by diclofenac acyl glucuronide, even though it obviously must associate closely with

this reactive metabolite to enable biliary excretion [116].

Summarized, the evidence supporting a role for reactive acyl glucuronides in ulceration is:

1) induction of diclofenac glucuronidation significantly increased small intestinal ulceration [115];

2) NSAIDs that do not show extensive glucuronidation and do not undergo extensive enterohpatic recirculation show reduced intestinal toxicity [110,117];

3) species with extensive enterohpatic circulation are more sensitive to the ulcerogenic effects of NSAIDs than others that do not excrete glucuronides into bile to the same extent.

Support for a role for enterobacteria and their endotoxins as important mediators of small intestinal ulceration comes from the attenuation of ulceration by antibiotic treatment [118] and the fact that germ-free rats are resistant to the gastrointestinal toxicity of NSAIDs [119]. These well known factors mean that a role for bacteria has often been fitted into other models of NSAID-induced gut injury. Gut flora alter the pharmacokinetics of acyl glucuronides presented to the small intestine via the bile. Bacterial β-glucuronidase facilitates the enterohpatic cycling by cleaving the glucuronide conjugates and allowing reabsorption of parent drug. This permits the re-uptake of the parent drug by the gut allowing enterohpatic circulation to re-expose the gut to metabolite. However, given: (a) the evidence for alternative mechanisms of injury, especially for those that include a direct role for free drug; and (b) that a mechanism by which the presence of adducts could cause ulceration is still unidentified, the evidence for a direct role for acyl glucuronides in gastrointestinal toxicity remains circumstantial.

3.3.6.4. Tubulin polymerization. The demonstration that the in vitro covalent modification of the ubiquitous eucaryotic protein tubulin by zomepirac acyl glucuronide and its isomers caused inhibition of tubulin polymerization is more evidence that a functional change in a protein could
have a role to play in the diverse ADR of acyl glucuronide forming drugs [120]. That zomepirac-modified tubulin was found in the livers of rats dosed with this drug opens up the possibility of in vivo ramifications. The tubulin–microtubule system is absolutely essential to a myriad of functions within cells that could explain various ADRs of these drugs. For example, hepatobiliary transport relies on functioning transport pathways and a breakdown of these could possibly trigger cholestasis. Also, as will be discussed (Section 3.3.6.6), use of certain NSAIDs has long been associated with a diminished risk of cancer. As the tubulin–microtubule system is essential for cell division, inhibition or stabilization of this dynamic system would perturb cell division. Recent work has found a modest role for valproic acid acyl glucuronide in inhibiting the assembly of tubulin in vitro [121]. That this relatively stable acyl glucuronide caused any inhibition was surprising, and the results suggested that both covalent and non-covalent interactions with tubulin and/or ‘microtubule-associated proteins’ were responsible for the effects.

3.3.6.5. Superoxide dismutase activity. Another protein shown to be functionally compromised after incubation with an acyl glucuronide is superoxide dismutase [122]. This in vitro study demonstrated the effects of preincubation with glucose, glucuronic acid and suprofen acyl glucuronide on reversible binding of model substrates to human serum albumin and on activity of superoxide dismutase. After incubation of superoxide dismutase with suprofen acyl glucuronide at a concentration of 5 mM for 14 days, enzyme activity was reduced to about 11% of control values. Suprofen acyl glucuronide was much more potent than either of the sugars (a similar level of inhibition was achieved with glucuronic acid using a 100-fold higher concentration). The study concluded that suprofen acyl glucuronide was much more efficient at non-enzymic glycation of albumin and superoxide dismutase than either glucuronic acid or glucose. However, as for the tubulin experiments, the relevance of highly selected in vitro experiments to the in vivo situation is unclear.

3.3.6.6. Antiproliferative effects in colorectal and other cancers?. The use of aspirin and other NSAIDs (especially sulindac) has long been associated with reduced risk of colorectal cancer. Several mechanisms have been advanced attempting to explain these observations [123,124], resulting in several hundred articles published on this topic in the last 10 years. None of these has considered a role for reactive acyl glucuronides, until recently [125]. Biliary excretion of reactive NSAID acyl glucuronides could be followed by their rearrangement into β-glucuronidase-resistant isomers, thus affording protection against intestinal β-glucuronidases, and facilitating transport through the small intestine to reach the site of the cancer in the colon [41]. Could the acyl glucuronide isomers be then taken up into cancer cells and covalently modify proteins (e.g. tubulin) critical in the cell replication process [125]? This question was addressed in vitro by incubating a number of acyl glucuronides with a human colon cancer cell line, but the study was unable to provide compelling evidence for direct involvement of acyl glucuronides/isomers in the observed antiproliferative effects, which could have originated from the NSAIDs themselves following hydrolysis of the acyl glucuronide/isomers. As well, there is evidence that non-carboxylate cyclo-oxygenase (COX)-2 specific inhibitors have efficacy in chemoprevention, and that COX-2 inhibition itself (rather than inhibition of COX-1 and -2) may have a critical role [126,127]. There is also evidence that certain NSAIDs exert their antiproliferative effects without necessarily inhibiting COX (sulindac sulfone is antiproliferative and does not inhibit either COX enzyme). In fact, both traditional and COX-2 specific NSAIDs seem to have antiproliferative effects in culture with both COX containing and COX null mutants. Therefore, both COX-dependent and -independent mechanisms seem to be at work. At least two mechanisms (and possibly more, see Ref. [128]) seem to affect the cell cycle in different ways.

Interestingly, the antitumor agents 5,6-dimethylxanthenone-4-acetic acid and flavone-8-acetic acid cause induction of cytokines (see earlier section on mycophenolate mofetil) and form acyl glucuronide metabolites [129–131]. 5,6-Dimethylxanthenone-
4-acetic acid also has been demonstrated to form covalent adducts both in vitro and in vivo [132]. Furthermore, a compound in clinical trials as an anti-carcinogenic agent (LGD1069, Targretin, a retinoid ‘X’ receptor-selective ligand or rexinoid) forms acyl glucuronide conjugates of both the parent compound and its hydroxy metabolite [133]. One could speculate that acyl glucuronides play an important but indirect role in the anticarcinogenic effects of NSAIDs by a mechanism akin to those of these agents. However, the present evidence does not support any direct role.

3.3.6.7. DNA adducts. Recent in vitro work has demonstrated damage to DNA (concentration-dependent decrease in the transfection efficiency in the M13 forward mutational assay) after incubation with gemfibrozil and clofibric acid acyl glucuronides [134]. No damage occurred after incubation with the parent drug, and 10 times less occurred after incubation with the endogenous glycating agent glucose-6-phosphate. This suggests a possible role for reactive acyl glucuronides in the genotoxicity of these hypolipidemics. However, previous work utilizing 14C-labeled clofibric and fenofibric acids administered to rats [135] clearly demonstrated covalent modification of liver nuclear proteins, but no such binding to DNA. While covalent interaction may not produce DNA-adducts stable enough to be identified, the possibility of non-covalent interaction causing the observed damage cannot be ruled out.

3.3.7. Individual susceptibility

As serious ADRs are so rare, the genesis of an idiosyncratic ADR of either the ‘metabolic/functional’ or ‘immune’ kinds would perhaps require an ‘unusual’ or ‘atypical’ component to the normal metabolic situation. This could be an abnormality in metabolism, drug or metabolite transport, receptor function, ion channels or possibly immune status. These differences may come about because of genetic polymorphism (absence of a gene, different genes {and therefore enzymes} or non-functional or overactive genes) or differences in expression of the same enzymes [136]. Pathological state (e.g. inflammation), exposure to other xenobiotics and hormones are known to change the activity and expression (as well as activity) of some enzymes.

Another reason why few people ever develop idiosyncratic ADRs may be that the vast majority become immunologically tolerant to drug-adducted proteins in a similar way to that demonstrated for other oral antigens [100,137,138], i.e. only the few who do not develop tolerance would get an ADR. This may come about because of polymorphism in one or more genes or because of the influence of environmental factors such as drugs or a concomitant infection or the effects of age.

As alluded to in previous sections, the initial events eliciting immune sensitization and therefore antibody production (or effector cell stimulation) in response to acyl glucuronide-forming drug therapy have not been verified, and antibody production often occurs in patients who do not later experience pathology. In fact, adverse events involving immune reactions are uncommon (although they make up a large proportion of ADRs when they do occur), and exposure to genetic and environmental factors may be necessary before toxicity develops. Competent metabolic and immune systems are likely also required for an immune-based toxicity.

3.3.8. Dose dependency

Contrary to the long-held notion that idiosyncratic ADRs are dose-independent, it seems that dosage may also be a factor in somehow determining whether or not an idiosyncratic ADR will occur. Drugs given at high mass doses are often associated with higher frequencies of idiosyncratic ADRs than those with lower mass dose regimens. Uetrecht [76] suggests that drugs given at doses less than 10 mg/day may not cause idiosyncratic ADR.

4. Conclusions

The in vivo disposition of carboxylate drugs forming reactive acyl glucuronides is complex. Fig. 2 provides a representation of the multiple cycles operative. A carboxylate drug may undergo a little excretion unchanged in urine or bile, and it may
form non-glucuronide metabolites. A major pathway can be the formation of the acyl glucuronide conjugate, which can undergo excretion in urine and bile. However, the acyl glucuronide also readily undergoes systemic and enterohepatic hydrolysis (catalyzed by β-glucuronidases, esterases, serum albumin and hydroxide ion) to reform the pharmacologically-active parent drug. The acyl glucuronide can also undergo hydroxide ion-catalyzed rearrangement via acyl migration and anomerization, to form isomers. The isomers are also available for excretion in urine and bile, and hydrolysis (with the same catalysts but excepting the β-glucuronidases) to reform the pharmacologically-active parent drug. The acyl glucuronide (via a transacylation mechanism) and its rearrangement isomers (via a glycation mechanism) can cause the covalent modification of endogenous proteins and other macromolecules. While these disposition pathways may be quantitatively minor, they may have significant biological impact by possibly initiating immune responses and cellular dysfunction, at least in a small population of susceptible individuals.

In terms of the future for carboxylate drugs forming acyl glucuronides as major metabolites, perspective needs to be maintained. It is worth looking at the existing situation. On the one hand, valproic acid is an established anti-epileptic agent forming an acyl glucuronide as its major metabolite. It is given in gram or hundred milligram doses daily. In spite of a record of human deaths measured in the low hundreds worldwide (since 1980), it is generally considered as a ‘safe drug’. Its chronic use in patients worldwide is enormous, and (under conditions of clinical awareness and biochemical surveillance for any sign of hepatotoxicity), its market share continues to expand. Valproic acid acyl glucuronide is still the least reactive drug acyl glucuronide yet investigated, and it has not been implicated in the pathogenesis of valproic acid-associated idiosyncratic hepatotoxicity. On the other hand, there are quite a number of older generation carboxylate drugs that form highly reactive acyl glucuronides (some with serious ADR attributed to them), which are still on the market (e.g. probenecid).

So what conclusions can be drawn from all this evidence? (As noted earlier, it should be remembered, in this context, that in vitro experiments finding biological consequences of acyl glucuronide-derived covalent modification of proteins have usually been specifically designed to amplify this possible outcome.) People taking carboxylate drugs that form reactive acyl glucuronides will form covalent drug–protein adducts, predominantly in the liver and blood compartments, with the gut and kidney compartments (the latter little discussed here) also being ‘targets’. While drug–protein adduct formation may be widespread (indeed it is most likely universal for carboxylate drugs forming even slightly reactive glucuronides), idiosyncratic ADR are rare, so a cause and effect supposition is not straightforward. This is despite the fact that there is much in vitro circumstantial evidence to suggest that reactive glucuronides, whether by adduct formation or by other mechanisms, can initiate idiosyncratic adverse reactions. Even though NSAIDs (especially) are used by such high numbers of people, adverse events may only occur in genetically or metabolically challenged individuals i.e. those with one or more predisposing factors. The fact that, of all the carboxylate drugs on the market, it is the NSAIDs that seem to be most represented in those that have been removed from the market because of idiosyncratic ADR is worth mentioning. This may be due to the frequency of use of NSAIDs (one of the most commonly used classes of drugs). It could also possibly be because of a generally higher level of acyl glucuronide reactivity in this class, or a higher dosage level for NSAIDs compared with other carboxylate drugs, or, again, a combination of factors.

While there is persuasive evidence supporting the notion that reactive acyl glucuronides could initiate idiosyncratic ADR, little direct evidence demonstrates a link between acyl glucuronide-derived covalent drug–protein adduct formation and adverse biological consequences. Until comprehensive molecular mechanisms that explain how ADRs are initiated by acyl glucuronide-derived covalent drug–protein adducts are established, a broadly-based toxic role for reactive acyl glucuronides binding to proteins remains uncer-
tain. In the meantime, development of carboxylate drugs based upon lower dose (more potent) agents that form lower yields of acyl glucuronides of lower relative reactivity seems likely to be the prudent approach to minimize the possibility of idiosyncratic ADR.

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