ELSEVIER

Teaser The association of reactive acyl-glucuronide drug metabolites with toxicity, particularly hepatotoxicity, is a concern in drug discovery and development, which this article puts into perspective in light of current knowledge.

Acyl glucuronide reactivity in perspective

Peter R. Bradshaw[1], Toby J. Athersuch[1], Andrew V. Stachulski[2] and Ian D. Wilson[1]

[1] Division of Systems Medicine, Department of Metabolism, Digestion and Reproduction, Imperial College London, Exhibition Road, South Kensington, London, SW7 2AZ, UK
[2] Robert Robinson Laboratories, Department of Chemistry, University of Liverpool, Liverpool, L69 7ZD, UK

Acyl glucuronidation is a common metabolic fate for acidic drugs and their metabolites and, because these metabolites are reactive, they have been linked to adverse drug reactions (ADRs) and drug withdrawals. However, alternative routes of metabolism leading to reactive metabolites (e.g., oxidations and acyl-CoA thioesters) mean that unambiguous proof that acyl glucuronides are toxic is lacking. Here, we review the synthesis and reactivity of these metabolites, and describe the use of molecular modelling and *in vitro* and *in vivo* reactivity assessment of acyl glucuronide reactivity. Based on the emerging structure-dependent differences in reactivity and protein adduction methods for risk assessment for acyl glucuronide-forming acid drugs or drug candidates in drug discovery/development are suggested.

Introduction

Glucuronidation is a common metabolic fate for a variety of drugs, and other xenobiotics and their oxidative metabolites. In general, glucuronidation is considered to be a detoxication reaction but, in the case of acidic drugs (or acidic metabolites of drugs produced via ester hydrolysis or oxidative metabolism), the formation of acyl glucuronides is a likely metabolic fate. Such an outcome is typical for, for example, nonsteroidal anti-inflammatory drugs (NSAIDs), many of which are associated with liver or gut toxicity. As a result of the propensity for these drugs to be metabolized to acyl glucuronides, and the long-recognized chemical reactivity of these metabolites [1–5], an association between acyl glucuronide reactivity and toxicity has been made, particularly for drug-induced liver injury (DILI). However, this association is a loose one and, despite decades of research, absolute proof is still lacking. Thus, although there is clear evidence for covalent binding to proteins for these metabolites, the situation is often complicated by the fact that there is often concomitant metabolism via oxidative routes that form reactive metabolites that can also lead to covalent binding. A good case in point is diclofenac, which is associated with a high level of ADRs, including DILI; there is also clear evidence from patients of circulating

Corresponding author: Wilson, I.D. (i.wilson@imperial.ac.uk)



Toby Athersuch is a senior lecturer at Imperial College, London. His research focus is on understanding xenobiotic metabolism in relation to risk assessment, and as a component of the human exposome. He has a long-standing interest in reactive metabolites and their role in drug-induced toxicity.



Peter Bradshaw studied for his BSc in Biochemistry from Sheffield Hallam University before undertaking PhD studies at Imperial College, London. Following defence of his doctoral thesis in predictive drug metabolism, he continued as an MRC ITTP Research Fellow combining *in vitro* studies, computational chemistry, and machine learning to study aspects of drug metabolism and toxicology.



Andrew Stachulski is a senior research fellow at the University of Liverpool, where he has worked since 2001 apart from a period in 2010˜2011 when he was on placement at Oxford University. He has published ~100 research papers, reviews, and patents with an H index of 25. The medicinal chemistry of glucuronides has been one of his major research interests for over 20 years.



Ian Wilson began researching acyl glucuronides in 1981 when working in the pharmaceutical industry. On leaving AstraZeneca (2012) he joined Imperial College, London, where he is currently a visiting professor of drug metabolism and molecular toxicology. The author, or co-author, of over 550 publications, he has received awards in separation and analytical science from the Royal Society of Chemistry and the Chromatographic Society. His research in drug metabolism focusses on mechanisms of adverse drug reactions, including acyl glucuronides.

1359-6446/© 2020 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
https://doi.org/10.1016/j.drudis.2020.07.009

adducts to human serum albumin (HSA) [6] in the absence of obvious toxicity, although CYP P450-related reactive metabolism is also significant [7]. Perhaps the best evidence for reactive acyl glucuronides being the cause of DILI is the fact that those with the fastest transacylation rates in buffer tend to be associated with the most compound withdrawals and/or black box warnings [8] However, such high rates of rearrangement might simply be pointing towards some other steric or electronic property of the molecule that makes it susceptible to other mechanisms of toxicity (i.e., the formation of reactive S-acyl CoA esters; e.g., [9–13]).

Whatever the case, the formation of ester glucuronides has attracted regulatory opprobrium and they are considered to be toxic *per se* regardless of whether this is in fact the cause. Such regulatory concerns were first highlighted in the US Food and Drug Administration (FDA) regulatory *Guidance on the Safety testing of Metabolites*, which essentially labelled acyl glucuronides as toxic [14]. In addition, a recent industry and regulatory publication [15] contained statements such as ' . . . *O*-glucuronides, *O*-sulfates, and quaternary N^+-glucuronides may be considered benign from a human safety perspective, animal coverage of acyl glucuronides could still be warranted because of reactivity concerns'. In addition to glucuronidation, the carboxylate function is subject to other types of conjugation, including to highly reactive *S*-acyl CoA conjugates, and these have been accorded little regulatory attention [12]. Indeed, in one study, it was noted that acyl glucuronides with a high acyl migration rate combined with a high daily dose, plus the presence of *S*-acyl CoA conjugates (or further downstream acyl CoA-derived metabolites) correlated with 'a higher probability to cause drug-induced liver injury' [10]. An alternative conclusion might be that it was the CoA metabolites that were the toxins and the acyl glucuronides innocent bystanders, a view reinforced by a recent study on *p*-alkyl-benzoyl-CoA conjugates [13].

So, despite many years of research the situation is still, in our view (and indeed that of many others), unresolved. There remains a lack of convincing evidence for the acyl glucuronides, as a class, being a cause of human DILI. Nevertheless, such opinions should be a spur to action, and it is the case that the finding of an acyl glucuronide as a metabolite of a carboxylic acid-containing drug presents a problem for both drug discovery and development. The same applies to compounds such as celecoxib, which, although not a carboxylic acid itself, acquires such a group as a result of oxidative metabolism and that metabolite goes on to form an acyl glucuronide [16].

The situation with respect to potential acyl glucuronide toxicity is made worse by a lack of any form of predictive animal model that can be used to test the hypothesis. In such a situation, the best that can be done until predictive models are available, or acyl glucuronides are removed from the toxin list, is to follow best practice, and a good example of this was recently provided by Smith *et al.* [3], who advanced a 'simplified paradigm' for the safety assessment of acyl glucuronides that provides valuable guidance for a pragmatic approach to dealing with this problematic class of reactive metabolites. Here, we highlight the current state of the art in certain areas of the field, make suggestions as to approaches to testing the hypothesis that acyl glucuronides are indeed toxic, and provide additional tools to aid decision making.

Synthetic aspects of acyl glucuronides

In their proposed simplified approaches to the safety assessment of these metabolites Smith *et al.* [3] stated that 'The instability of acyl glucuronides in aqueous solution poses formidable challenges in establishing appropriate tests for an unqualified acyl glucuronide . . . .' which is indeed the case. They went on to state that the 'Isolation or chemical synthesis of acyl glucuronides is possible, but extremely difficult in any quantity' and, although agreeing with the first part of this sentence, we would suggest that advances in synthetic methods for these metabolites have advanced to the stage where the chemical synthesis of these compounds in quantity is feasible using accessible and published procedures based on the selective acylation method. This versatile and efficient approach to acyl glucuronide synthesis relies on the kinetic anomeric effect, through which 1β-acylation is favoured over 1α- in a mildly basic medium. Moreover, this method requires only monoprotection, typically using either allyl [17] or benzyl [18] D-glucuronate. In this way, the synthesis of several acyl glucuronides has been demonstrated, including relevant drug examples, in an efficient two-step synthesis (see [19] for phenylacetic acid examples). In addition, the field is not static, and new chemical, biochemical, and biosynthetic approaches continue to be developed, as outlined next.

Progress in synthetic methods for acyl glucuronides

The use of fully protected carbohydrate intermediates for acyl glucuronide synthesis was revived by Nagao *et al.* [20]. Thus, conversion of known allyl glucuronate **1** [21] to the tetra-allyloxycarbonyl derivative **2**, followed by anomeric carbonate hydrolysis, afforded hemiacetal **3** (Fig. 1).

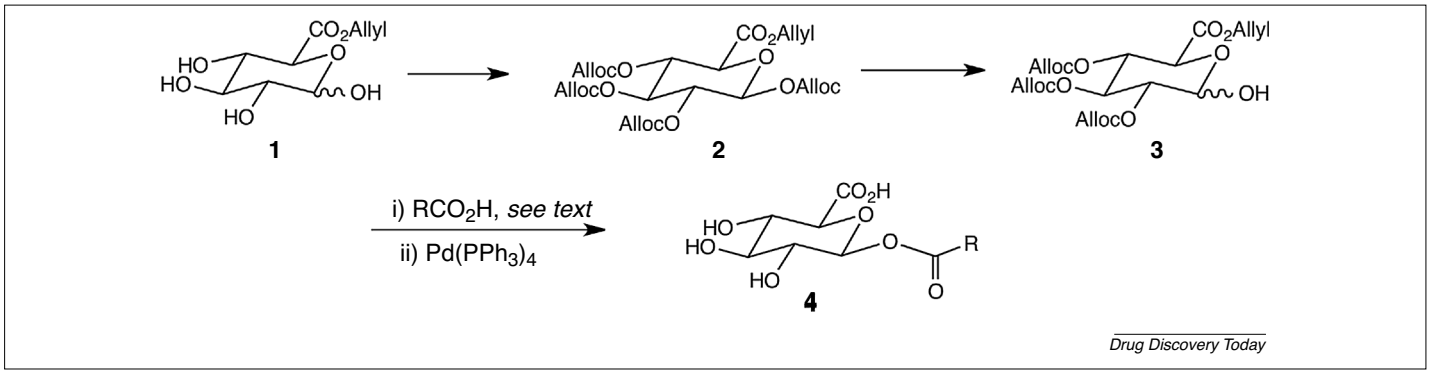


FIGURE 1

Acyl glucuronide synthesis via a tetraallyl intermediate.

Various carboxylic acids, following appropriate activation, were coupled to **3** with excellent β:α selectivity, affording the desired acyl glucuronide **4** after exhaustive Pd(0) deprotection (Fig. 2). The authors recommend the chloropropenylamine derivative **5** as an activator in this mode (Fig. 2). Gram quantities of the acyl glucuronide of PPAR-γ-agonist **6** were made in this way (Fig. 2).

The selective acylation method was recently refined by using a chiral base catalyst [22]. Thus, acylation of carboxylic acids with anomeric hemiacetal sugars, catalysed by a single enantiomer of chiral base **7**, afforded the corresponding glycosyl esters as α- or β-anomers. Using (*S*)-**7**, the β-product was exclusively formed; using (*R*)-**7**, the α-anomer was preferred, with lower selectivity (Fig. 2).

Notably, (*S*)-ibuprofen could be esterified without epimerisation by this method. Tetrabenzyl glucose **8** was used in most cases (Fig. 2), but other pyranose sugars could be used; the concept has not yet been tested for acyl glucuronidation.

Recent applications of existing synthetic methods

Enzyme-based methods and cell preparations

The chemoenzymatic approach of Baba *et al.* [23] is still used. Thus, alkylation of carboxylic acid **9** with the appropriate anomeric bromosugar afforded intermediate **10** exclusively as the 1β-anomer (Fig. 2). After some optimisation, sequential enzymatic hydrolysis of the acetates, then the methyl ester, afforded the desired acyl glucuronide of **9** (Fig. 2); additionally, the authors determined the degradation kinetics of both (*2R*)-and (*2S*)-isomers of the product.

Biotransformation continues to be used for direct glucuronidation of drug molecules, including acyl glucuronides. Recently a

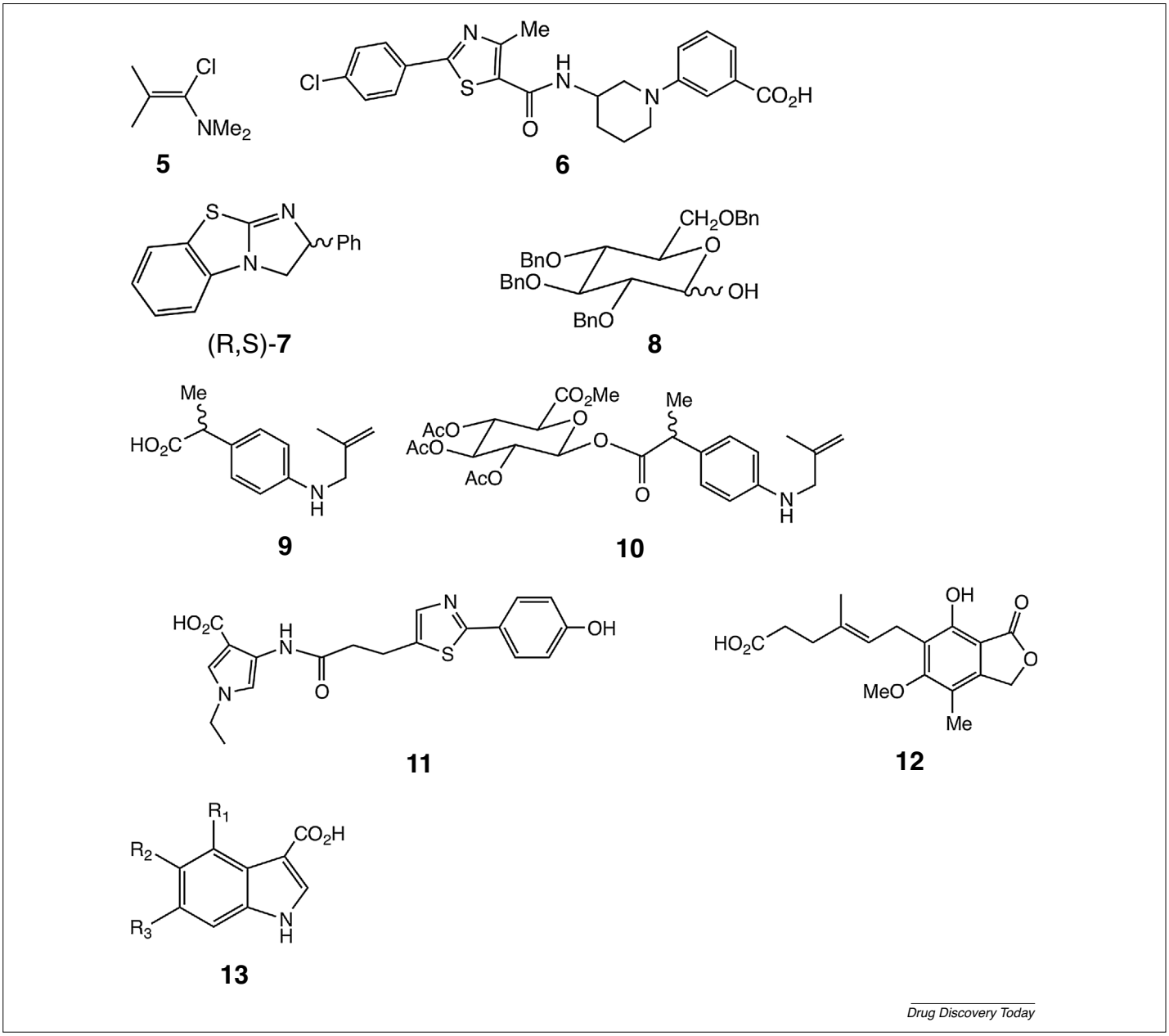


FIGURE 2

Structures 5–13.

Streptomyces strain SANK 60895 [24] was used for the syntheses of the acyl glucuronides of the lipolysis inhibitor **11** and mycophenolic acid **12** (Fig. 2), although, in both cases, preparative high-performance liquid chromatography (HPLC) was needed to separate the acyl glucuronides from the simultaneously produced aryl glucuronides. Finally, human microsomal preparations are still used, as in the series of indole 3-carboxylic acid derivatives **13** (Fig. 2), three of which were converted to their acyl glucuronides in this way [25].

Selective acylation

Several examples of the use of the selective acylation method, using either allyl or benzyl glucuronate, have appeared; carboxylic acids **14**–**21** converted to their acyl glucuronides in this way are summarised in Figure 3.

In Figure 3, **14** is itself an oxidative metabolite of amine **14a**, a controlled psychoactive [26]. Fasiglifam **15** is an agonist of the free fatty acid receptor (FFAR1, also known as GPR40); both **15** and a significant metabolite **16** were converted to their acyl glucuronides [27]. In a related medicinal chemistry programme [28], **17** was identified as a candidate versus GPR40 and was also extensively metabolised as its acyl glucuronide.

The series of four acyl glucuronides derived from phenylpropionic acid derivatives **18**, related to ibuprofen (R_1, R_2 = H or Me; both *R*- and *S*-enantiomers of the monomethyl derivative), together with acids **19** and **20**, were prepared specifically for a proteomic study with purified HSA (see further discussion later) [29]. Extending this concept further, the known acyl glucuronide of diclofenac **21** was prepared [6] for a study of albumin adducts found in patients taking diclofenac (see further discussion later).

Assessment of the reactivity of acyl glucuronides

Transacylation of acyl glucuronides in buffer and plasma

The ester bond of the 1β-anomer is liable to nucleophilic attack through two mechanisms: hydrolysis by hydroxide ions resulting in the parent aglycone and α/β-glucuronic acid, and attack by adjacent hydroxyl groups on the glucopyranose ring, which results in intramolecular transacylation. As is now well appreciated, the percentage of each reaction differs according to the structure of the aglycone and the matrix in which the metabolite is present [30–32]. Within the buffer, the transacylation reaction dominates, and hydrolysis to the free aglycone at physiological pH can be relatively minor (although the balance between the two outcomes changes rapidly in more alkaline solutions). However, in plasma, hydrolysis of the acyl glucuronide metabolite is the major reaction [33,34]. The transacylation rate of the original acyl glucuronide and its various transacylated forms in plasma also proceeds at different rates, as shown by studies on the reactivity of diflunisal acyl glucuronide (DFAG) isomers [35]. When the individual 1-, 2-, 3-, and 4-*O*-acyl positional isomers were incubated (at ~55 μg DF equivalents/mL) with HSA (40 mg/mL, pH 7.4, 37 °C) initial half-lives of 53, 75, 61, and 26 min were measured, respectively. Whereas the 1-*O*-acyl isomer was subject to hydrolysis, the 2-, 3-, and 4-*O*-acyl isomers, especially the latter, were more prolific at forming covalent adducts of HSA adducts compared with the 1-*O*-acyl form. Thus at 2 h, the 1-, 2-, 3-, and 4-*O*-acyl positional isomers had formed adducts with HSA accounting for 2.4, 8.2, 13.7, and 36.6% of the amount originally present in the incubation, respectively [35].

However, studies in aqueous buffer are technically simpler to perform and are more widely used than plasma to assess the

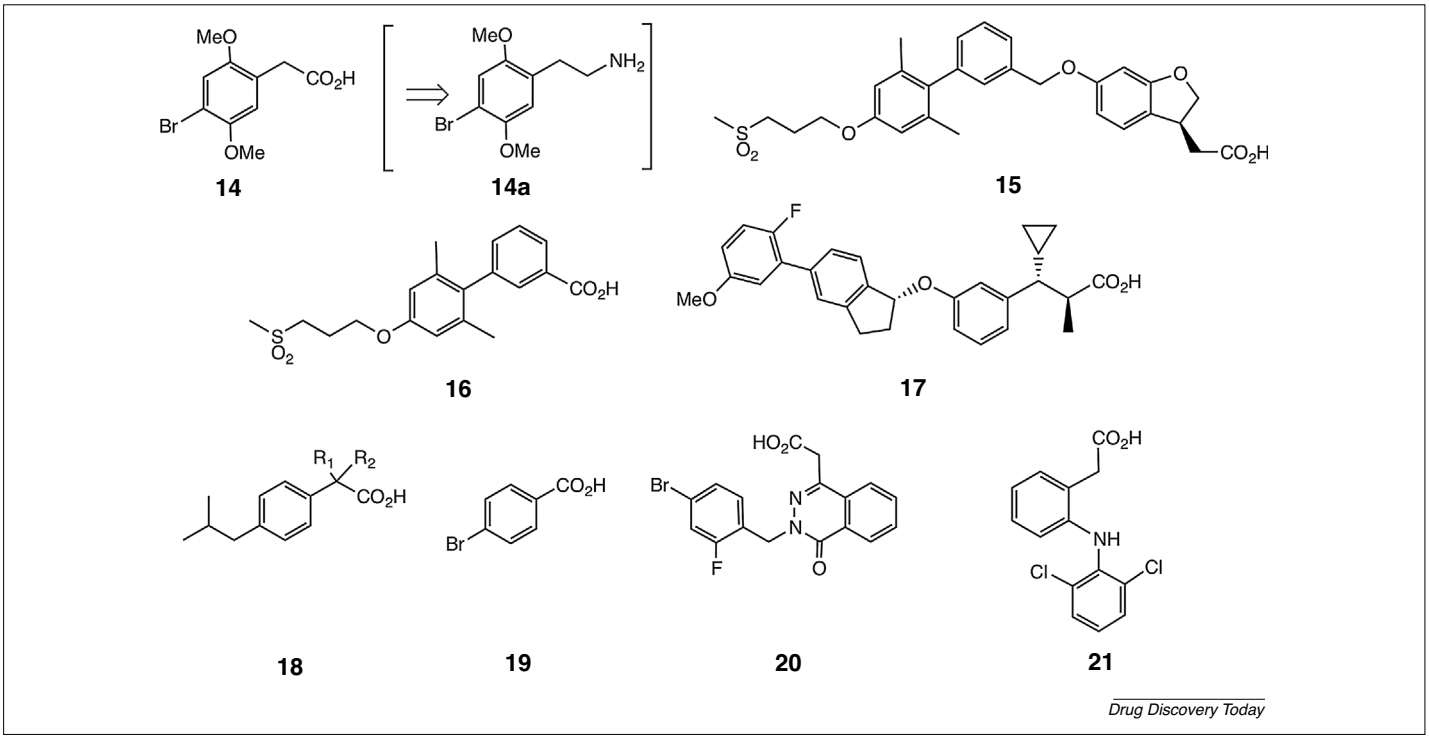


FIGURE 3

Carboxylic acids converted to their acyl glucuronides by the selective acylation method.

potential reactivity of acyl glucuronides. In buffer, at pH ~7.4, after the initial transacylation to the 2β-anomer, subsequent transacylation and anomerisation reactions result in the appearance of multiple α/β-anomers until an equilibrium mixture is attained. Measuring the disappearance of the 1β-anomer over time because of transacylation and hydrolysis reactions *in vitro* has been utilised as a measure of the overall reactivity of the acyl glucuronide and has been termed the degradation rate (k_d). Two analytical techniques have been predominantly used for determining the k_d: proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and HPLC with ultraviolet (UV) or mass spectrometric (MS) detection. Differences in rates of degradation determined by each technique have been observed; for example, the k_d for benzoic acid differed by approximately ninefold, being 0.039 h^{-1} by ^{1}H NMR spectroscopy [36] and 0.35 h^{-1} by HPLC-UV [37] Deuterated solvents have previously been shown to decrease the rate of degradation of acyl glucuronides [38], which might account for the differences observed between rate constants determined by ^{1}H NMR spectroscopy and HPLC-UV/MS. This might be because of deuterium exchange with the hydroxyl hydrogen of the glucopyranose ring, resulting in a decreased rate of transacylation. However, the ability to perform such studies in largely protonated solvents, including water, should limit these differences in degradation rate determination by ^{1}H NMR spectroscopy [33,34].

The aglycone structure has been shown to affect the k_d value through both electronic and steric factors. In general, the inclusion of electron-withdrawing groups (EWG) on the aromatic ring adjacent to the ester bond enhances the rate of degradation, and the inverse is observed for substitution with electron-donating groups (EDG). This was clearly observed with the difference in degradation rate of a series of *para*-substituted benzoic acids investigated by Vanderhoeven *et al.* [36,39]. 4-Nitrobenzoic acid had a k_d of 0.71 h^{-1}, whereas replacing the electron withdrawing nitro-group with EDGs, such as 4-methoxy- or 4-propoxy-substiuents, decreased the rate of reaction to 0.013 h^{-1} and 0.007 h^{-1}, respectively, determined by ^{1}H NMR spectroscopy.

Differences in the rate of degradation were also observed because of the position of the group around the benzoic acid ring, measured by HPLC-UV. Baba and Yoshioka [37] investigated the degradation of the acyl glucuronides of the *ortho*-, *meta*-, and *para*-derivatives of chlorobenzoic acid, methylbenzoic acid, and phenylbenzoic acid, along with the *ortho*- and *para*- derivatives of methoxybenzoic and trifluoromethylbenzoic acid. The degradation of the *meta*- derivatives 3-chlorobenzoic acid, 3-methylbenzoic acid, and 3-phenylbenzoic acid proceeded faster than their equivalent *para*- metabolites for each series of compounds. The *ortho*- species 2-methylbenzoic acid and 2-phenylbenzoic acid were the slowest of their respective series, highlighting the role of steric factors in the rate of degradation. 2-Chlorobenzoic acid degraded slightly faster than the *para*- derivative, with a k_d of 1.21 h^{-1} compared with 0.61 h^{-1} for 4-chlorobenzoic acid. Similarly, 2-methoxybenzoic acid was determined to have a k_d of 0.146 h^{-1}, which is faster than the *para*- derivative with a rate of 0.082 h^{-1}. By contrast, 2-trifluoromethylbenzoic acid degraded more slowly than the *para*- derivative, with a k_d of 0.234 h^{-1} compared with 1.79 h^{-1} for 4-trifluoromethylbenzoic acid [40]. These examples demonstrate that a mixture of electronic and steric factors governs the rate of degradation of the acyl glucuronide metabolites.

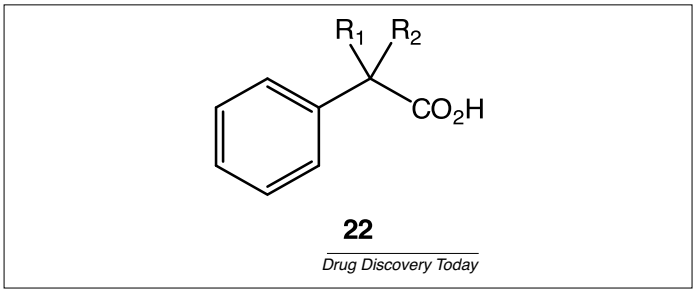


FIGURE 4
Structure 22.

The influence of steric hindrance has also been investigated using a series of phenylacetic acid **22** (R_1, R_2 = H or Me; (*R*)- and (*S*)-diastereoisomers of the monomethyl derivative; Fig. 4) and ibuprofen **18** analogues (Fig. 3) [19,33]. Substitution of methyl groups to the α carbon increased the stability of the acyl glucuronide conjugate with the dimethyl derivatives of both series, α,α′-dimethyl phenylacetic acid and 2-(4-isobutylphenyl)-2-methylpropanoic acid (bibuprofen) having the slowest reaction times, k_d 0.029 h^{-1} and 0.025 h^{-1}, respectively, determined by ^{1}H NMR spectroscopy. In contrast the unsubstituted derivatives, phenylacetic acid and ibufenac had more rapid reaction rates, 2.353 h^{-1} and 0.516 h^{-1}, respectively. In the case of the single methyl substitution to give the (*R*)-diastereoisomers, (*R*)-α-methyl phenylacetic acid and (*R*)-ibuprofen (k_d 0.903 h^{-1} and 0.472 h^{-1}, respectively) degradation was faster than the corresponding (*S*)-diastereoisomers, (*S*)-α-methyl phenylacetic acid and (*S*)-ibuprofen (k_d 0.405 h^{-1} and 0.184 h^{-1}, respectively). This pattern in degradation between diastereoisomers has been observed with multiple aryl propionic acid acyl glucuronide metabolites, including benoxaprofen [41] carprofen [42], fenoprofen [43], ketoprofen [44], and naproxen [45,46]. The (*S*)-diastereoisomers degrade at approximately half the rate of the (*R*)-diastereoisomers (leading to the easily remembered phrase 'R for rapid, S for slow'). Here, we have discussed the two major classes of aglycone documented in the literature (benzoic acid and aryl acetic acid derivatives); the reader is directed to [47] for further discussion of acyl glucuronides reactivity on a diverse range of aglycone structures.

Target sites for covalent binding of acyl glucuronides on albumin

Although transacylation/hydrolysis rates are useful indicators of reactivity, clearly the 'proof of the pudding' of reactivity with biomolecules, such as proteins, relies on direct evidence. Whereas phenolic glucuronides, for example, are unreactive glycosidic bonds, acyl glucuronides can react with proteins through both transacylation and intramolecular acyl migration to form glycation and glycosylation products (Fig. 5). Advances in proteomic techniques enable the detection of both protein adducts and their sites and nature. Although relatively few detailed studies have been performed, data from both *in vitro* and *ex vivo* studies have been obtained and illustrate what can be achieved.

There is good, longstanding, evidence for the covalent binding of acyl glucuronides to proteins *in vivo* and these modified proteins

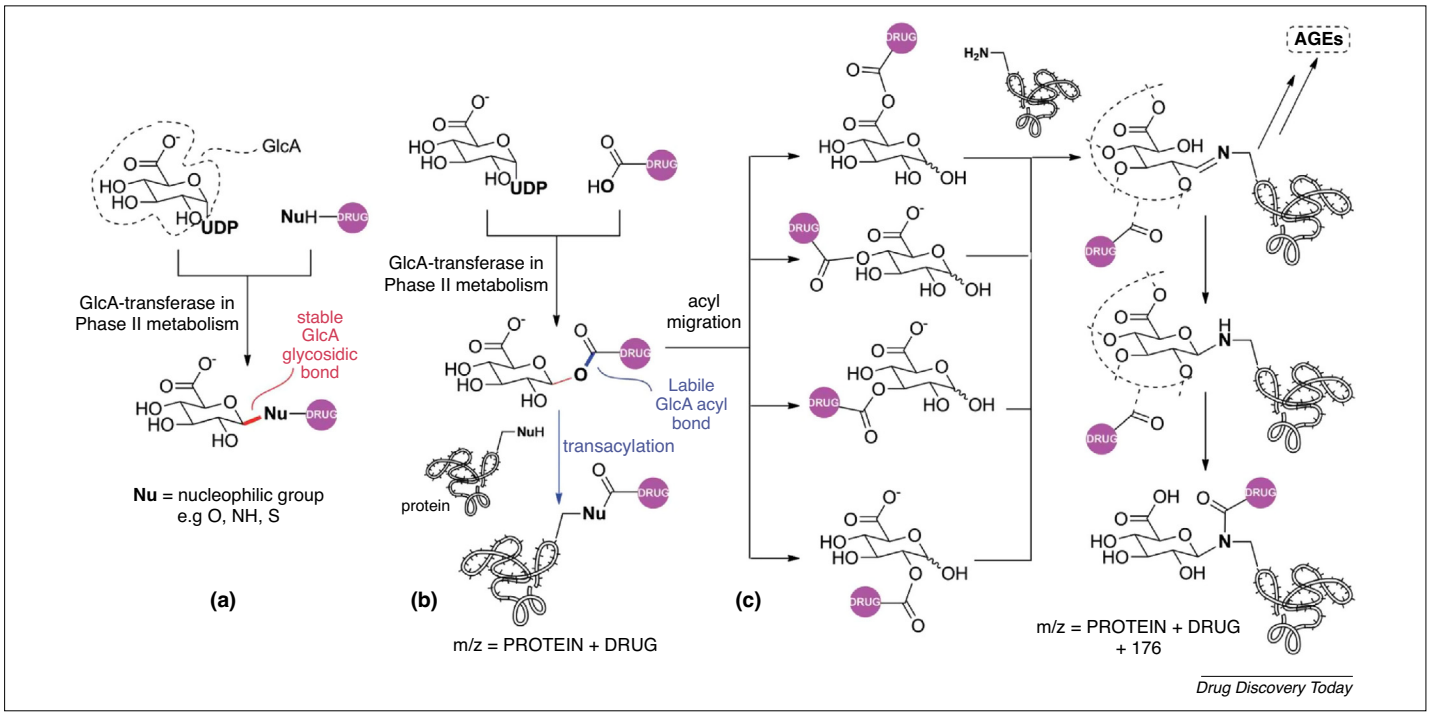


FIGURE 5

The formation, reactions and putative products of nucleophilic groups on drugs. **(a)** Glucuronidation of nucleophilic groups (Nu) in drugs during conjugative metabolism. A production of stable glucuronide glycosidic bonds (e.g., with phenolic groups). **(b)** Glucuronidation to produce acyl glucuronides, which can then react with proteins via transacylation or **(c)** intramolecular acyl migration and then react to form glycated and glycosylated proteins. Reproduced, with permission, from [28].

then eliciting an immune response. An example is valproic acid, which has its acyl glucuronide conjugate as a major metabolite. Protein adducts of valproic acid were detected in plasma obtained from both *in vitro* incubations and from patients with epilepsy treated with the drug [48]. By using ELISA (developed with the aid of HSA–drug adducts formed following incubation with a mixture of the transacylated glucuronides anomers), the immunoreactivity of plasma from 57 patients was tested. Of these, nine patients were found to have low, but measurable, amounts of antibodies to HSA modified in this way. However, the low titres were taken to indicated that the drug–protein adducts had, in line with low clinical valproate hypersensitivity, similarly low immunogenicity. That said, this early study nicely illustrates the progression from *in vitro* studies, which reveal the potential for HSA modification, to *ex vivo* studies to better determine the biological response (or lack of it) in the patient. Several studies were able to pinpoint the sites of covalent adduction on HSA, such as those describing the binding of tolmetin and benoxaprofen acyl glucuronides [49,50]. In the case of tolmetin [49], the adducts produced when its acyl glucuronide was incubated *in vitro* with HSA were identified as either being linked via the glucuronic acid primarily to Lys-199 and, in smaller amounts, to Lys-195 and -525 or direct linkage of tolmetin itself to lysine (Lys-199 and 541), serine (Ser-220, 232, and 480) and Arg-222 residues. In addition, there was indirect evidence for binding of tolmetin acyl glucuronide to Lys-541, and binding of tolmetin to Arg-521. These results established that binding of acyl glucuronides to various nucleophilic sites on proteins occurred mainly via imine formation, retaining the glucuronic acid, or alternatively via nucleophilic displacement of the glucuronic acid moiety. The same group used similar methods to study the covalent adducts formed when the acyl glucuronide of benoxaprofen was incubated with HSA [50]. Unlike tolmetin, Lys-159 was found to be the major site of these benoxaprofen-derived adducts, indicating that there were compound-specific differences in the adduct-binding profiles.

Continuing advances in proteomic technologies have revealed more details concerning the nature and extent of protein modification. Recent studies showed the effect of the structure of the acyl glucuronide upon adduct formation following *in vitro* incubation of a range of acyl glucuronides [ibufenac, (*R*)-/(*S*)-ibuprofen, bibuprofen, *p*-bromobenzoic acid and ponalrestat] with HSA at concentrations typical of those encountered in therapy [29]. This study showed that, for most of the compounds tested, adduct formation via both transacylation and glycosylation could occur (although glycosylation predominated for *p*-bromobenzoic acid acyl glucuronide), with small amounts of dual adducts formed for ibufenac and (*R*)- and (*S*)-ibuprofen (Fig. 6). The resulting protein product ratios that were obtained related to the degree of α-substitution to the acyl group, and a lack of α-substitution correlated with higher reactivity as determined by HSA adduction. As discussed earlier, *in vitro* degradation in buffer of the phenylacetic acid [19] and ibuprofen series [33] is related to α-substitution and there might be a correlation between *in vitro* k_d and the type of protein adducts formed. Of particular interest is that the pattern of protein modification by the acyl glucuronides varied greatly with structure. However, as seen with the earlier studies, Lys-137, 195, 199, 436, and 525 were modified, with Lys-137 and 436 showing glycosylation and transacylation-derived adducts.

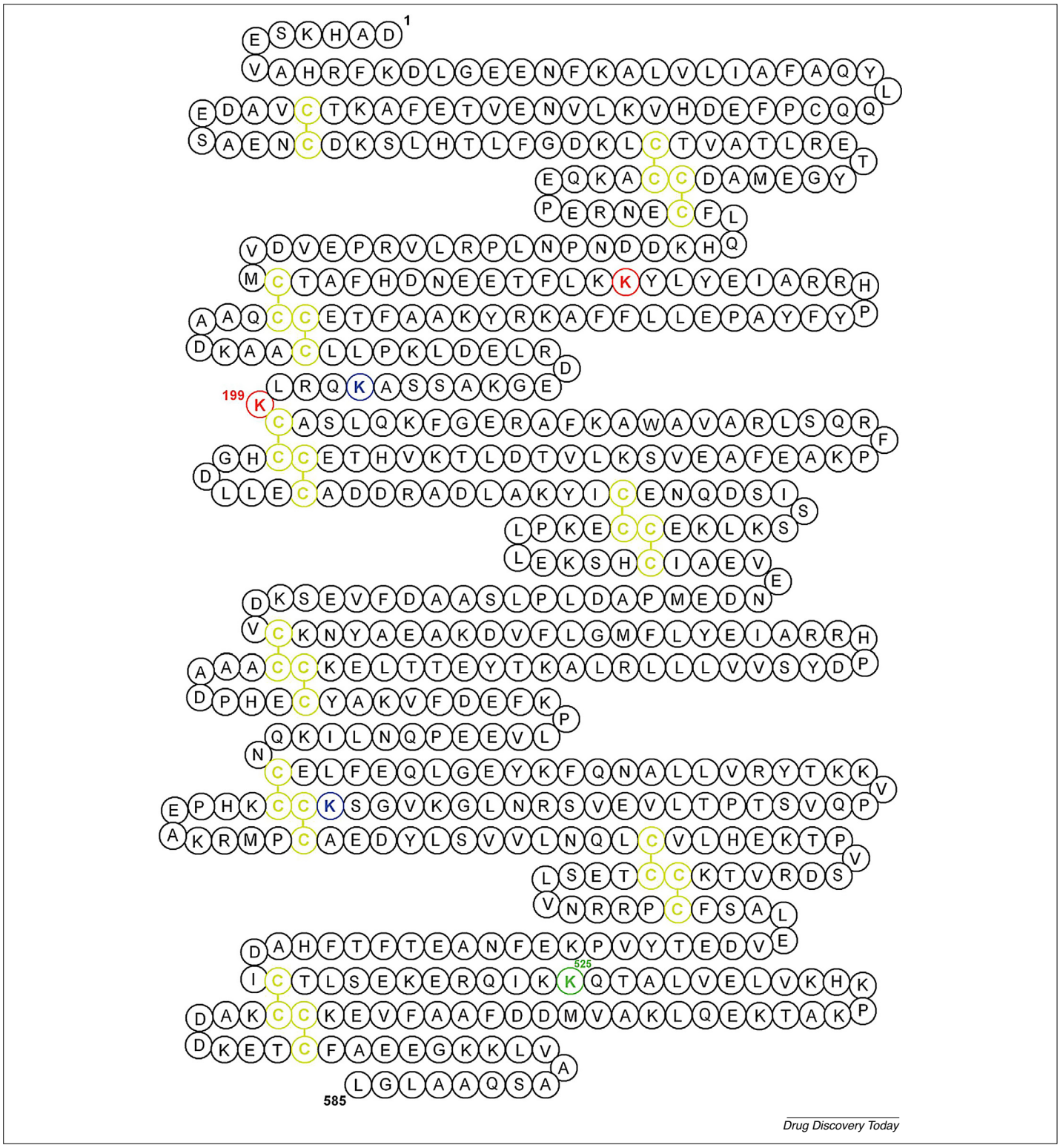


FIGURE 6

The sites and reactivity of acyl glucuronide metabolites with human serum albumin (HSA). The sequence map shows the primary amino acid sequence with disulfide bonds shown in yellow. Reaction sites are shown for in red for glycosylation, transacylation sites are indicated in blue, whereas Lys-525, which had both glycosylation and transacylation reactivity, is in green. Reproduced, with permission, from [28].

Studies undertaken on the reaction of synthetic diclofenac acyl glucuronide, and then patient-derived HSA, revealed a similarly interesting and complex pattern of adduction [6]. *In vitro* incubations of the diclofenac metabolite with HSA at a molar ration of 50:1 showed both glycation and transacylation at Lys-137, 190, 195, 199, 351, 432, 436, 525, and 451 with, in addition, glycation at Lys-162. However, at lower glucuronide:HSA ratios (e.g., 0.1:1), only Lys-190, 199, and 432 adducts were identified. When HSA

from six patients was analysed with the same methodology, modification of seven lysine residues was observed, with seven *N*-acylations and three acyl glucuronide glycations. The transacylation to Lys-195 was seen for all the patients, whereas one patient had all seven lysine adducts. The *in vitro* studies were not entirely accurate predictors of the *in vivo* result, with adducts of Lys-137 and 351 not detected. However, as the authors noted for these patients, none of whom showed evidence of diclofenac-related hypersensitivity, 'albumin adduction is not inevitably a causation of hypersensitivity to carboxylate drugs or a coincidental association' [6].

Quantitative structure–property relationship models

Multiple quantitative structure–property relationship (QSPR) models have been developed to identify and quantify the structural features of the aglycone, which might be useful for predicting the rate of degradation. Given the nucleophilic nature of the transacylation and hydrolysis reactions, the electron density at the carbonyl carbon is likely to be key for predicting k_d values. Vanderhoeven *et al.* [36,39] investigated electronic effects on the rate of degradation of acyl glucuronide metabolites with a series of *para*-substitution benzoic acid acyl glucuronides, as discussed earlier. Hammett constants showed good correlation ($R^2 = 0.95$, $N = 10$), with the logarithm of the degradation rate constant (log k_d). Baba and Yoshioka [37] identified a similar correlation with a series of *meta*- and *para*-substituted benzoic acids ($R^2 = 0.98$, $N = 10$). Partial atomic charges on the carbonyl carbon of the ester bond calculated using different population analysis schemes were investigated as descriptors for predicting log k_d. Calculations at the Hartree–Fock level, with the STO-3 G basis set using Mulliken population analysis, resulted in the closest correlation ($R^2 = 0.96$, $N = 10$) with log k_d of the population analysis schemes investigated [39]. Similar results were observed by Yoshioka and Baba [40] at the B3LYP/6-31G* level of theory with water simulated as the solvent using natural population analysis for the charge on the hydrogen ($R^2 = 0.94$, $N = 10$) and oxygen atoms ($R^2 = 0.95$, $N = 10$).

Vanderhoeven *et al.* [39] also investigated the correlation between measured ^{13}C NMR shifts of the carbonyl carbon and the degradation rate. The chemical shift for the parent benzoic acid ($R^2 = 0.78$, $N = 9$), ethyl ester ($R^2 = 0.78$, $N = 9$) and acyl glucuronide ($R^2 = 0.79$, $N = 9$) showed similar correlations with the log k_d. Similar results were observed by Baba and Yoshioka [37] with the *ortho*-, *meta*- and *para*- benzoic acid aglycone series ($R^2 = 0.84$, $N = 16$) and their corresponding acyl glucuronide ($R^2 = 0.88$, $N = 16$). These authors also performed further linear regression with their *meta*- and *para*-substituted benzoic acids and identified the experimental measured pK_a ($R^2 = 0.94$, $N = 10$) and calculated pK_a ($R^2 = 0.97$, $N = 10$) as useful descriptors for predicting log k_d [40]. In addition to single linear regression, multiple linear regression models combining electronic (NMR chemical shifts, partial atomic charge, and calculated pK_a) and steric descriptors (NMR chemical shifts and Taft E parameters) were developed ($R^2 = 0.90$–0.92, $Q^2 = 0.78$–0.89, $N = 18$) for the series of 18 *ortho*-, *meta* and *para*-substituted benzoic acids (see Table 6 in [40]). Similar regression models were developed using electronic and steric descriptors for a separate series of aralkyl carboxylic acids [51]. Potter *et al.* [52] also developed a partial least squares regression model using ^{13}C NMR chemical shifts and Sterimol steric parameters of methyl esters as a proxy for acyl glucuronide activity.

The models discussed earlier focused on the calculation of simple descriptors of the parent aglycone, ethyl ester, or conjugate to predict the rate of degradation. Berry *et al.* [19] used a more sophisticated approach by modelling the initial transacylation reaction using density functional theory (DFT). They identified the transition structures for four phenylacetic acid **22** acyl glucuronide derivatives and calculated the activation energy between the ground state and transition structure using B3LYP/6-31++G(d,p)//B3LYP/6-31 G(d,p) model chemistry. The calculated activation energy showed good correlation with k_d ($R^2 = 0.98$, $N = 4$), as did the energy of the lowest unoccupied molecular orbital (E_{LUMO}) ($R^2 = 0.90$, $N = 4$). The activation energy of the transacylation reaction accounts for both electronic and steric elements that affect the rate of degradation and has potential to be a useful descriptor for accurate prediction of degradation. However, because of the larger size of the molecular system (acyl glucuronide conjugate) compared with simply optimising the aglycone structure and the need to identify the transition structures, this method is more time and computationally intensive than methods discussed earlier. Currently, this methodology has examined a limited subset of acyl glucuronides and its application is currently being extended by examining more acyl glucuronide and acyl glucoside structures. Acyl glucosides are a related set of metabolites that undergo transacylation and hydrolysis reaction analogous to their glucuronide counterparts [53,54]. The above methodology was recently successfully applied to the equivalent acyl glucoside series [54] of phenylacetic acid studied by Berry *et al.* [19].

For QSPR model development in general, the larger the data set the better; the models discussed earlier were built on small congeneric series, which limits their application. Recently, Tugcu and Sipahi [55] developed a global model using a large diverse training set of aglycone structures. Their data set contained 67 acyl glucuronides (collated in [47]), and 3100 descriptors were calculated for each aglycone structure using multiple cheminformatic software. Feature selection was performed with the genetic algorithm method before the development of linear regression models. From the feature selection, the four-descriptor regression model reported had the number of quaternary carbons (nCq), ring complexity index (RCI), burden eigenvalues (BELe6), and edge adjacency indices (ESpm07d) as key features ($R^2 = 0.69$, $Q^2 = 0.57$, $N = 67$). As expected, the model had a combination of descriptors that capture steric and electronic effects.

However, the data set used for the model comprised half-life values for the compounds determined by either NMR spectroscopy or HPLC-MS; for example, values for the phenylacetic acid **22** series [phenylacetic acid, (R)-/(S)-2-phenylpropionic acid and 2-methyl-2-phenylpropionic acid] were determined by NMR spectroscopy [19,47,55], whereas half-lives for fenclofenac, levofloxacin, meclofenamic acid, and repaglinide were determined by HPLC-MS/MS [8,47,55] [as a note, it appears there is an incorrect value for the phenylacetic acid half-life (1.05 h), whereas 0.29 h was reported in the original article [19]]. As discussed earlier, the degradation rate determined for a compound can differ greatly depending on the analytical technique used, especially if NMR spectroscopy was performed in D_2O. Another potential limitation of this model is that half-live values for diastereoisomers were

averaged for the aglycone and entered as a single data point [e.g., (*R*)-/(*S*)-2-phenylpropionic acid, (*R*)-/(*S*)-ibuprofen, (*R*)-/(*S*)-ketoprofen, etc.]. As discussed earlier, the diastereoisomers follow the general pattern of the (*R*)-diastereoisomers degrading approximately twice as fast as the (*S*)-diastereoisomers. The aryl propionic acid class of molecules has been studied extensively in drug development (include many NSAIDs) and, therefore, for accurate prediction of k_d and half-lives, it would be more appropriate to distinguish between the diastereoisomers as separate data points. This distinction is often difficult using molecular descriptors based on the aglycone structure because the values are often the same or similar for the (*R*)- and (*S*)-diastereoisomers. Despite the disadvantage of the added time and computation, the methodology presented by Berry *et al.* [19] and Bradshaw *et al.* [54] has the advantage that it can distinguish between the diastereoisomers because of differences in the geometry (and, therefore, energy) of the (*R*)- and (*S*)-conjugates. In addition, the authors examined the correlation of descriptors individually with subclasses of acyl glucuronide (R = 0.78–>0.99; see Table 4 in [55]). Examination of individual classes of aglycone decreased the sample size (*N* = 5–30) compared with the global model, which was a limiting factor for the earlier models discussed thet were developed by other authors [19,36,37,39,40,51,52].

Based on the current published *in vitro* data, there is a trade-off between developing a model on a large data set (which, therefore, includes multiple classes of aglycone) resulting in lower accuracy, or alternatively developing a specific model for a class of conjugates (e.g., benzoic acids or aryl acetic acids), limiting the application of the model and potentially resulting in overfitting because of the small training set. Augmentation of the *in vitro* kinetic data available in the literature for acyl glucuronides with a larger range of aglycone structures, with care taken to ensure the same analytical technique, was used to have a defined endpoint, would be beneficial to the development of accurate predictive models.

Evaluating the risk posed by acyl glucuronides: model systems for hypothesis testing

Although the potential hazard posed by acyl glucuronides is clear, and an assessment of sorts can be made using simple measure, such as transacylation rates in buffer, obtaining a predictive evaluation of risk is more problematic. This is especially the case when there are other, competing, routes for toxification, such as reactive oxidative metabolism or *S*-acyl-CoA thioester formation [56]. However, a toolbox of *in vitro, in vivo, in silico*, and *ex vivo* studies is available that, although by no means fully developed, enables a more nuanced approach to predicting risk if used sensibly.

In vitro *systems*

Although perhaps of limited value, measuring the rate of transacylation in buffer is simple to undertake, can be performed on limited amounts of material (as might be obtained from *in vitro* or animal studies), and can act as a flag for the need to perform additional studies. We would suggest that the first of these would be incubation with proteins, such as HSA, to assess the potential for covalent binding. The sites and relative amounts of covalent modification can then be assessed using proteomic techniques, as outlined earlier. If reactivity against target proteins appears high, then absolute amounts of covalent binding can be determined via the use of radiolabelled drug. However, as noted in the Introduction, metabolism of carboxylic acid-containing drugs to both acyl glucuronides and reactive *S*-acyl-CoA thioesters occurs widely, and both types of metabolite bind to protein. Indeed, the latter have been shown to provide ~40–70 times the level of covalent binding to proteins compared with acyl glucuronides [12] A plausible hypothesis is then that acyl glucuronidation is acting as 'a canary in the mine', and is merely a surrogate marker for *S*-acyl-CoA thioester biosynthesis/reactivity, and that the latter is the real toxin. In addition, both types of reactive conjugate might be irrelevant if the real cause is oxidative bioactivation. However, methodology for assessing the relative contributions of both oxidative and conjugative biotransformations, and capable of distinguishing between all three metabolic fates, has been developed using human liver microsomes [12]. Here, microsomal preparations were supplemented with either NADPH (for oxidative metabolite production), uridine 5′-diphosphoglucuronic acid (UDPGA) (for acyl glucuronide formation), or CoA (to allow production of *S*-acyl-CoA thioesters). This system provides a readily implemented means for the separate study of the biosynthesis and reactivity of each type of metabolite. When applied to the investigation of ibuprofen, tolmetin, ibufenac, fenclozic acid, tienilic acid, suprofen, and zomepirac, all seven drugs formed acyl glucuronides, but no covalent binding to protein was detected as a result. By contrast, this methodology demonstrated that the CoA conjugates of ibuprofen, ibufenac, fenclozic acid, and tolmetin were highly reactive and, indeed, the highest levels of covalent binding achieved were seen with ibuprofenyl-CoA and ibufenacyl-CoA (1000 and 8600 pmol drug eq/mg protein, respectively) [12]. This observation is interesting because it is well known that racemic (*R*)-/(*S*)-ibuprofen is subject to significant conversion of the inactive (*R*)- form to the active (*S*)-ibuprofen and that the formation of acyl-CoA thioesters is central to the chiral inversion (which is also seen for many other racemic profens). Via their acyl-CoA thioesters, these profens can be further metabolised into lipids, such as hybrid triglycerides, and accumulated in adipose tissue and cell membranes [57]. In addition, they can affect lipid metabolism in a variety of ways [56]. Therefore, the reason for the lower covalent binding seen for ibuprofen versus ibufenac might not necessarily be because of reduced formation but might reflect the near tenfold difference in the reactivity of their acyl-CoA thioesters. Given that ibufenac was rapidly withdrawn from clinical use, whereas ibuprofen remains a popular, and relatively safe, over-the-counter NSAID, it is tempting to speculate that, had these relative covalent binding potency measurements been available at the time, the latter would have been selected over ibufenac for further development. In the same study, both suprofen and tienilic acid showed much higher reactivity for their oxidative metabolites (250 pmol drug eq/mg protein each) than was attributable to either acyl glucuronides or CoA conjugates.

More recently Shang *et al.* [11] used a similar approach to investigate the reactivity of a drug candidate, MK-8666 (tromethamine) designed to be a selective GPR40 agonist for use in type 2 diabetes mellitus, but the development of which was terminated as a result of apparent DILI. Covalent binding studies were undertaken using liver microsomes, as well as rat and human hepatocytes, to determine the role, if any, of reactive metabolites in the observed DILI. These studies showed that the drug was converted to

an acyl glucuronide in human hepatocytes and both acyl glucuronide and taurine conjugates in those of rat. Irrespective of species, the amounts of protein binding were similar. Proteolysis enabled the identification of a range of transacylated products to lysine, serine, and cysteine, as well as glycation adducts. The latter was produced via the rearrangement of the 1-*O*-β-acyl form of the glucuronide to ring-opened aldehydes capable of condensing with lysine in the proteins as imine adducts. Notably, increased amounts of protein binding were detected when the microsomes were supplemented with either CoA or ATP compared with that seen with microsomal incubations fortified with UDPGA. When glutathione was added to the MK-8666 incubation, the amount of binding attributable to CoA thioester formation was reduced by >40%. Therefore, this study provides another demonstration of the ability of this methodology to separate and quantify the various sources of covalent protein modification produced as the result of exposure to acidic drugs (or their metabolites).

Obviously, more advanced cellular models, such as hepatocytes, also have potential for providing this type of information, including systems using inhibitors of CYP450, or the HepG2 cell line, where P450 activity is low. In the case of glucuronidation, the natural product (-)-borneol can provide some inhibition of acyl glucuronide formation, but this is not always complete. For example, in the case of the acyl glucuronide of valproic acid, hepatocytes incubated with 1 mM (-)-borneol clearly reduced production of the conjugate (but did not eliminate it) without any effect on the chosen markers of toxicity [58]. In the case of diclofenac acyl glucuronide production (used as a positive control in the study), no effect was detected on the production of its acyl glucuronide. By contrast, hepatocyte investigations on the drug fasiglifam (TAK-875) using a radiolabelled form of the drug attributed the significant covalent binding observed to a reactive acyl glucuronide and/or an acyl-CoA thioester [59] This conclusion was made on the basis that co-incubation of hepatocytes with CYP450 inhibitor 1-aminobenzotriazole (ABT) had little effect on covalent binding, whereas (-)-borneol reduced it significantly (40%). Diclofenac, used as a positive control, had its covalent binding reduced by both ABT and borneol. Although hepatocytes are clearly useful, it would appear that the easiest screen to introduce initially would be one based on hepatic microsomes, which offer the potential to investigate each of these potential metabolic fates separately. Hepatocytes might then be used for subsequent studies.

In vivo *systems*

The availability of efficient methods for the synthesis of gram quantities of acyl glucuronides removes one practical barrier to their *in vivo* testing, but several others remain, a major one being stability. If orally administered acyl glucuronides are unlikely to be absorbed to any extent, they will probably not survive because of chemical stability issues and the effects of bacterial glucuronidase (although this can be ameliorated). This means that not only will the test compounds have to be administered intravenously, but they may also require long infusions to maintain any level of exposure. Uptake into target organs, such as the liver, might also be limited and, taken together, these factors do not make this a particularly attractive approach.

However, where the metabolic fate of a drug, or drug candidate, is solely through acyl glucuronide formation, the extent of exposure and adverse effects, together with the amount of covalent binding, is relatively easy to determine (although interpretation might still be problematic) using conventional studies. Where glucuronidation is the major route of metabolism models, such as that established in the mouse, investigating the renal toxicity of zomepirac [60] can be useful. This study involved the use of both the esterase inhibitor tri-*O*-tolyl phosphate (esterases, in addition to glucuronidases, hydrolyse acyl glucuronides [61]) and the glutathione biosynthesis inhibitor BSO to explore the mechanism of tissue damage. In cases where there is a mixture of oxidative metabolism to reactive metabolites, acyl-CoA thioester and acyl glucuronide formation *in vivo* evaluation is obviously more problematic, and more difficult to dissect than *in vitro* systems. A partial solution can be found in the use of, for example, mice in a study of diclofenac-induced DILI, where (-)-borneol was used to inhibit glucuronidation [62] and both a reduction in DAG concentrations and markers of hepatic injury were seen compared with controls. Alternatively, genetically modified mice can be used, such as the hepatic reductase null (HRN^{TM}) mouse, where hepatic CYP 450s are essentially inactive, eliminating reactive metabolite formation by this route. However, conjugative metabolism still occurs in the livers of HRN^{TM} mice and both glucuronide and amino acid conjugates are formed. The latter is important because the amino acid conjugates (most often to glycine in human and taurine in mouse), produced in the mitochondria, require the biosynthesis of an acyl-CoA thioester [63] However, such thioester formation does not always result in amino acid conjugation [e.g., acyl-CoA thioesters of (*R*)-ibuprofen appear to be recycled predominantly as (*S*)-ibuprofen, not the glycine conjugate in humans]. When compared with normal C57Bl6 mice, which produce a mixture of oxidative metabolites and (mainly) taurine, ether, and (small amounts of) acyl glucuronide conjugates, HRN^{TM} mice form essentially only the acyl glucuronide and taurine conjugates. Thus, by comparing metabolic profiles in both conventional and HRN^{TM} mice, it is possible to demonstrate that metabolism is either largely confined to the liver, or also subject to extrahepatic metabolism (e.g., [64–66]). If, for example, in studies in normal and HRN^{TM} mice, the bulk of metabolism can be assigned to the liver, then a better idea of the overall metabolic fate could be obtained using chimeric liver humanized mice. In these animals, most mouse hepatocytes are replaced with metabolically competent human-derived cells. With drugs such as fenclozic acid [67] or diclofenac [68], where metabolism is largely via the liver, humanized mice showed that large amounts of the acyl glucuronide conjugate (accompanied by smaller quantities of the transacylated glucuronides) were produced, with taurine conjugates representing only a minor component [67,68]. By contrast, taurine conjugates represented a larger proportion of the total metabolite profile in normal mice, suggesting that these humanized mice give a better reflection of the likely exposure of humans to acyl glucuronide [67,68].

Ex vivo *studies in humans*

Clearly, as described earlier, there is unequivocal evidence for covalent binding to, for example, HSA in the circulation of patients receiving drugs, such as diclofenac [6] and valproic acid [48]. Equally clearly, the presence of these circulating acyl glucuronide-modified proteins does not automatically indicate the

likelihood for a subsequent ADR. However, using proteomic techniques, it should be feasible to assess this covalent binding for a range of acyl glucuronide-forming drugs in both animals and humans. From this, it might be possible to determine whether there is a predictive QSAR that can distinguish between toxic compounds, perhaps based on the sites and types of modification, and those that are not, which could be used in risk assessment. It should also be possible to obtain a measure of the immunogenicity of these modified proteins.

Concluding remarks and perspective

Despite many years of speculation and investigation, there is still no clear and unambiguous evidence that acyl glucuronides are toxic as a result of reactivity. Certainly, many compounds that do form these conjugates have been associated with DILI, and a significant subset of these have been withdrawn. However, as has often been pointed out, many DILI-causing compounds (e.g., diclofenac), which form acyl glucuronides with short *in vitro* half-lives, are also subject to oxidative metabolism and *S*-acyl Co-A conjugate formation, both of which also produce reactive metabolites. However, although it can be argued that the presence of the carboxylic acid group is key, and whether it is the acyl glucuronide or *S*-acyl CoA-metabolite that is the source of the covalent binding is a detail, it is difficult to disentangle these from the reactive oxidative metabolites. However, synthetic methods are now available that can produce high-purity acyl glucuronides in hundreds of milligram to gram quantities if required to enable further testing, if only the appropriate tests can be devised. A variety of *in vitro*, *in vivo*, and *ex vivo* models are also available that could provide insight into likely risk.

With advances in computational power, molecular systems can be modelled in greater detail. As discussed earlier, DFT has been utilised to model the intramolecular transacylation transition structures of a limited number of acyl glucuronide metabolites. Further investigations into a larger series of acyl glucuronides will be useful for determining whether this methodology is effective for QSPR development. In addition, modelling of large systems, including proteins, has become a growing field in recent decades. Calculations using hybrid quantum mechanics/molecular mechanics (QM/MM) have been applied to study the active site of enzymes and ligand interactions with proteins and DNA (see Table 2 in [69] for a list of studies applying QM/MM to biomolecules). This technique could be used to study the mechanism of protein adduct formation of acyl glucuronides and *S*-acyl-CoA thioesters, potentially aiding the identification of the mechanism(s) of toxicity. The key point that needs resolution at the moment, as highlighted by Boelsterli in 2002 [56], is probably that 'more work is needed to provide a causal link between protein-reactive acyl glucuronides and acyl-CoA thioesters and the rare and unpredictable idiosyncratic drug reactions in humans', or indeed to demonstrate whether there is a genuine link between either (or both) of these reactive metabolites and toxicity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

A personal MRC Fellowship to P.B. is gratefully acknowledged.

References

1 Faed, E.M. (1984) Properties of acyl glucuronides: implications for studies of the pharmacokinetics and metabolism of acidic drugs. *Drug Metab. Rev.* 15, 1213–1249
2 Stachulski, A.V. *et al.* (2006) Acyl glucuronides: biological activity, chemical reactivity, and chemical synthesis. *J. Med. Chem.* 49, 6931–6945
3 Smith, D.A. *et al.* (2018) Safety assessment of acyl glucuronides—a simplified paradigm. *Drug Metab. Dispos.* 46, 908–912
4 Regan, S.L. *et al.* (2010) Acyl glucuronides: the good, the bad and the ugly. *Biopharm. Drug Dispos.* 31, 367–395
5 Van Vleet, T.R. *et al.* (2017) Acyl gucuronide metabolites: implications for drug safety assessment. *Toxicol. Lett.* 272, 1–7
6 Hammond, T.G. *et al.* (2014) Mass spectrometric characterization of circulating covalent protein adducts derived from a drug acyl glucuronide metabolite: multiple albumin adductions in diclofenac patients. *J. Pharmacol. Exp. Ther.* 350, 387–402
7 Boelsterli, U. (2003) Diclofenac-induced liver Injury: a paradigm of idiosyncratic drug toxicity. *Toxicol. Appl. Pharmacol.* 192, 307–322
8 Sawamura, R. *et al.* (2010) Predictability of idiosyncratic drug toxicity risk for carboxylic acid-containing drugs based on the chemical stability of acyl glucuronide. *Drug Metab. Dispos.* 38, 1857–1864
9 Darnell, M. and Weidolf, L. (2013) Metabolism of xenobiotic carboxylic acids: focus on coenzyme A conjugation, reactivity, and interference with lipid metabolism. *Chem. Res. Toxicol.* 26, 1139–1155
10 Lassila, T. *et al.* (2015) Safety assessment of acyl glucuronides—a simplified paradigm. *Chem. Res. Toxicol.* 28, 2292–2303
11 Shang, J. *et al.* (2020) Bioactivation of GPR40 agonist MK-8666: formation of protein adducts in vitro from reactive acyl glucuronide and acyl CoA thioester. *Chem. Res. Toxicol.* 33, 191–201
12 Darnell, M. *et al.* (2015) Significantly different covalent binding of oxidative metabolites, acyl glucuronides, and S-acyl CoA conjugates formed from xenobiotic carboxylic acids in human liver microsomes. *Chem. Res. Toxicol.* 28, 886–896
13 Laue, H. *et al.* (2017) p-Alkyl-benzoyl-CoA conjugates as relevant metabolites of aromatic aldehydes with rat testicular toxicity-studies leading to the design of a safer new fragrance chemical. *Toxicol. Sci.* 160, 244–255
14 FDA (2016) *Safety Testing of Drug Metabolites*. FDA
15 Luffer-Atlas, D. and Atrakchi, A. (2017) A decade of drug metabolite safety testing: industry and regulatory shared learning. *Expert. Opin. Drug Metab. Toxicol.* 13, 897–900
16 Paulson, S.K. *et al.* (2000) Metabolism and excretion of [^{14}C]celecoxib in healthy male volunteers. *Drug Metab. Dispos.* 28, 308–314
17 Perrie, J.A. *et al.* (2005) Effective synthesis of 1β-acyl glucuronides by selective acylation. *Org. Lett.* 7, 2591–2594
18 Bowkett, E.R. *et al.* (2007) Efficient synthesis of 1β-O-acyl glucuronides via selective acylation of allyl or benzyl d-glucuronate. *Tetrahedron* 63, 7596–7605
19 Berry, N.G. *et al.* (2009) Synthesis, transacylation kinetics and computational chemistry of a set of arylacetic acid 1β-O-acyl glucuronide. *Org. Biomol. Chem.* 7, 2525–2533
20 Nagao, M. *et al.* (2016) The practical synthesis of β-acyl glucuronides by using allyl 2,3,4-tri-(O-allyloxycarbonyl)-d-glucuronate and 1-chloro-N,N,2-trimethyl-1-propenylamine. *Tetrahedron Lett.* 57, 3339–3343
21 Juteau, H. *et al.* (1997) A convenient synthesis of β-acyl glucuronides. *Tetrahedron Lett.* 38, 1481–1484
22 Ohnuki, T. *et al.* (2019) Practical one-step glucuronidation via biotransformation. *Bioorg. Med. Chem. Lett.* 29, 199–203
23 Baba, A. *et al.* (2018) Chemo-enzymatic synthesis, structural and stereochemical characterization, and intrinsic degradation kinetics of diastereomers of 1-β-O-acyl glucuronides derived from racemic 2-{4-[(2-methylprop-2-en-1-yl)amino]phenyl} propanoic acid. *ACS Omega* 3, 4932–4940
24 Wang, H.-Y. *et al.* (2017) Chiral catalyst-directed dynamic kinetic diastereoselective acylation of anomeric hydroxyl groups and a controlled reduction of the glycosyl ester products. *Org. Lett.* 19, 508–511

25 Ryder, T.F. *et al.* (2018) Acyl glucuronide metabolites of 6-chloro-5-[4-(1-hydroxycyclobutyl)phenyl]-1H-indole-3-carboxylic acid (PF-06409577) and related indole-3-carboxylic acid derivatives are direct activators of adenosine monophosphate-activated protein kinase (AMPK). *J. Med. Chem.* 61, 7273–7288
26 Kanamori, T. *et al.* (2017) Synthesis and analysis of glucuronic acid-conjugated metabolites of 4-bromo-2,5-dimethoxyphenethylamine. *J. Forensic Sci.* 62, 488–492
27 Ackerson, T. *et al.* (2019) Mechanistic investigations of the liver toxicity of the free fatty acid receptor 1 agonist fasiglifam (TAK875) and its primary metabolites. *J. Biochem. Mol. Toxicol.* 33, e22345
28 Pio, B. *et al.* (2019) Design, synthesis and biological evaluation of indane derived GPR40 agoPAMs. *Bioorg. Med. Chem. Lett.* 29, 1842–1848
29 Monrad, R.N. *et al.* (2014) Dissecting the reaction of Phase II metabolites of ibuprofen and other NSAIDS with human plasma protein. *Chem. Sci.* 5, 3789–3794
30 Hasegawa, J. *et al.* (1982) Apparent intramolecular acyl migration of zomepirac glucuronide. *Drug. Metab. Dispos.* 10, 469–473
31 Spahn-Langguth, H. and Benet, L.Z. (1992) Acyl glucuronides revisited: Is the glucuronidation process a toxification as well as a detoxification mechanism? *Drug. Metab. Rev.* 24, 5–47
32 Smith, P.C. *et al.* (1985) Irreversible binding of tolmetin glucuronic acid esters to albumin *in vitro*. *Drug Metab. Dispos.* 13, 110–112
33 Johnson, C.H. *et al.* (2010) Integrated HPLC-MS and (^{1})H-NMR spectroscopic studies on acyl migration reaction kinetics of model drug ester glucuronides. *Xenobiotica* 40, 9–23
34 Karlsson, E.S. *et al.* (2010) High-performance liquid chromatography/mass spectrometric and proton nuclear magnetic resonance spectroscopic studies of the transacylation and hydrolysis of the acyl glucuronides of a series of phenylacetic acids in buffer and human plasma. *Rapid Commun. Mass Spectrom.* 24, 3043–3051
35 Dickinson, R.G. and King, A.R. (1991) Studies on the reactivity of acyl glucuronides—II: interaction of diflunisal acyl glucuronide and its isomers with human serum albumin in vitro. *Biochem. Pharmacol.* 42, 2301–2306
36 Vanderhoeven, S.J. *et al.* (2004) NMR and QSAR studies on the transacylation reactivity of model 1beta-O-acyl glucuronides. I: design, synthesis and degradation rate measurement. *Xenobiotica* 34, 73–85
37 Baba, A. and Yoshioka, T. (2009) Structure–activity relationships for degradation reaction of 1-β-O-acyl glucuronides: kinetic description and prediction of intrinsic electrophilic reactivity under physiological conditions. *Chem. Res. Toxicol.* 22, 158–172
38 Mortensen, R.W. *et al.* (2001) S-Naproxen-β-1-O-acyl glucuronide degradation kinetic studies by stopped-flow high-performance liquid hromatography-^{1}H NMR and high-performance liquid chromatography-UV. *Drug. Metab. Dispos.* 29, 375–380
39 Vanderhoeven, S.J. *et al.* (2004) Nuclear magnetic resonance (NMR) and quantitative structure-activity relationship (QSAR) studies on the transacylation reactivity of model 1beta-O-acyl glucuronides. II: QSAR modelling of the reaction using both computational and experimental NMR. *Xenobiotica* 34, 889–900
40 Yoshioka, T. and Baba, A. (2009) Structure–activity relationships for the degradation reaction of 1-β-O-acyl glucuronides. Part 2: electronic and steric descriptors predicting the reactivity of 1-β-O-acyl glucuronides derived from benzoic acids. *Chem. Res. Toxicol.* 22, 1559–1569
41 Spahn, H. *et al.* (1989) Procedures to characterize in vivo and in vitro enantioselective glucuronidation properly: studies with benoxaprofen glucuronides. *Pharm. Res.* 6, 125–132
42 Georges, H. *et al.* (2000) In vitro stereoselective degradation of carprofen glucuronide by human serum albumin. Characterization of sites and reactive amino acids. *Chirality* 12, 53–62
43 Volland, C. *et al.* (1991) Stereoselective degradation of the fenoprofen acyl glucuronide enantiomers and irreversible binding to plasma protein. *Drug. Metab. Dispos.* 19, 1080–1086
44 Skordi, E. (2005) Kinetic studies on the intramolecular acyl migration of β-1-O-acyl glucuronides: application to the glucuronides of (R)- and (S)-ketoprofen, (R)- and (S)-hydroxy-ketoprofen metabolites, and tolmetin by 1H-NMR spectroscopy. *Xenobiotica* 35, 715–725
45 Bischer, A. *et al.* (1995) Stereoselective binding properties of naproxen glucuronide diastereomers to proteins. *J. Pharmacokinet. Biopharm* 23, 379–395
46 Mortensen, R.W. *et al.* (2002) Stereospecific pH-dependent degradation kinetics of R- and S-naproxen-beta-l-O-acyl-glucuronide. *Chirality* 14, 305–312
47 Camilleri, P. *et al.* (2018) The influence of physicochemical properties on the reactivity and stability of acyl glucuronides. *Xenobiotica* 48, 958–972
48 Williams, A.M. *et al.* (1992) Studies on the reactivity of acyl glucuronides—III: glucuronide-derived adducts of valproic acid and plasma protein and anti-adduct antibodies in humans. *Biochem. Pharmacol.* 43, 745–755
49 Ding, A. *et al.* (1995) Reactivity of tolmetin glucuronide with human serum albumin. Identification of binding sites and mechanisms of reaction by tandem mass spectrometry. *Drug. Metab. Dispos.* 23, 369–376
50 Qiu, Y. *et al.* (1998) Mechanisms for covalent binding of benoxaprofen glucuronide to human serum albumin. *Drug. Metab. Dispos.* 26, 246–256
51 Baba, A. and Yoshioka, T. (2009) Structure–activity relationships for the degradation reaction of 1-β-O-acyl glucuronides. Part 3: electronic and steric descriptors predicting the reactivity of aralkyl carboxylica acid 1-β-O-acyl glucuronides. *Chem. Res. Toxicol.* 22, 1998–2008
52 Potter, T. *et al.* (2011) In silico prediction of acyl glucuronide reactivity. *J. Comput. Aided Mol. Des.* 25, 997–1005
53 Iddon, L. *et al.* (2011) Synthesis of a series of phenylacetic acid 1-β-O-acyl glucosides and comparison of their acyl migration and hydrolysis kinetics with the corresponding acyl glucuronides. *Org. Biomol. Chem.* 9, 926–934
54 Bradshaw, P.R. *et al.* (2020) Kinetic modelling of acyl glucuronide and glucoside reactivity and development of structure–property relationships. *Org. Biomol. Chem.* 18, 1389–1401
55 Tugcu, G. and Sipahi, H. (2018) QSPR modelling of in vitro degradation half-life of acyl glucuronides. *Xenobiotica* 49, 1007–1014
56 Boelsterli, U. (2002) Xenobiotic acyl glucuronides and acyl CoA thioesters as protein-reactive metabolites with the potential to cause idiosyncratic drug reactions. *Curr. Drug. Metab.* 3, 439–450
57 Mayer, J.M. *et al.* (1994) Interactions of anti-inflammatory 2-arylpropionates (Profens) with the metabolism of fatty acids: in vitro studies. *Int. J. Tissue React.* 16, 59–72
58 Surendradoss, J. *et al.* (2014) Evaluation of in situ generated valproyl 1-O-β-acyl glucuronide in valproic acid toxicity in sandwich-cultured rat hepatocytes. *Drug Metab. Dispos.* 42, 1834–1842
59 Otieno, M.A. *et al.* (2018) Fasiglifam (TAK-875): Mechanistic investigation and retrospective identification of hazards for drug induced liver injury. *Toxicol. Sci.* 163, 374–384
60 Iwamura, A. *et al.* (2016) Zomepirac acyl glucuronide is responsible for zomepirac-induced acute kidney injury in mice. *Drug Metab. Dispos.* 44, 888–896
61 Fukami, T. and Yokoi, T. (2012) The emerging role of human esterases. *Drug Metab. Pharmacokinet.* 27, 466–477
62 Oda, S. *et al.* (2017) Toxicological role of an acyl glucuronide metabolite in diclofenac-induced acute liver injury in mice. *J. Appl. Toxicol.* 37, 545–553
63 Knights, K.M. *et al.* (2007) Amino acid conjugation: contribution to the metabolism and toxicity of xenobiotic carboxylic acids. *Expert Opin. Drug Metab. Toxicol.* 3, 159–168
64 Pickup, K. (2012) The hepatic reductase null mouse as a model for exploring hepatic conjugation of xenobiotics: application to the metabolism of diclofenac. *Xenobiotica* 42, 195–205
65 Pickup, K. *et al.* (2014) The metabolic fate of [14C]-fenclozic acid in the hepatic reductase null (HRN) mouse. *Xenobiotica* 44, 164–173
66 Grimsley, A. *et al.* (2014) A comparison of the metabolism of midazolam in C57BL/6J and hepatic reductase null (HRN) mice. *Biochem. Pharmacol.* 92, 701–711
67 Ekdahl, A. *et al.* (2018) The metabolic fate of fenclozic acid in chimeric mice with a humanized liver. *Arch. Toxicol.* 92, 2819–2828
68 Wilson, C.E. *et al.* (2018) The pharmacokinetics and metabolism of diclofenac in chimeric humanized and murinized FRG mice. *Arch. Toxicol.* 92, 1953–1967
69 Senn, H.M. and Thiel, W. (2009) QM/MM methods for biomolecular systems. *Angew. Chem. Int. Ed.* 48, 1198–1229