New Perspectives on Acyl Glucuronide Risk Assessment in Drug Discovery: Investigation of In vitro Stability, In situ Reactivity, and Bioactivation

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Abstract: Background: Acyl glucuronides of xenobiotics have been a subject of wide interest from the pharmaceutical industry with respect to biochemical reactivity, hepatic disposition, and enterohepatic circulation. The reactivity and lack of stability of an acyl glucuronide for a clinical candidate could pose major developability concerns. To date, multiple in vitro assays have been published to assess the risk associated with acyl glucuronides. Despite this fact, the translation of these findings to predicting clinical safety remains poor.

Methods: In the present investigation, we aimed to provide simplified in vitro strategy to understand the bioactivation potential of acyl glucuronides of 10 commercial, carboxylic acid containing drugs that have been categorized as “safe,” “warning,” or “withdrawn” with respect to their marketed use. Acyl migration was measured as a function of the number of peaks observed in LC-MS analysis. In addition, we carried out reactive intermediate trapping studies with glutathione and methoxylamine to identify the key intermediates in the transacylation bioactivation and glycation pathways, respectively. We also conducted reaction phenotyping with recombinant UDP-glucuronosyltransferase (UGT) Supersomes® to investigate if the formation of acyl glucuronides could be linked to specific UGT isoform(s).

Results: Our results were in line with reported values in the literature. Our assay could be used in discovery research where half-life calculation completely eliminated the need to chemically synthesize the acyl glucuronide standard for risk assessment. We captured our results for risk assessment in a flow chart to simplify the various complex in vitro techniques historically presented.

Conclusion: While the compounds tested from “withdrawn” and “warning category” all formed the glutathione adduct in buffer, none from “safe” category formed the glutathione adduct. In contrast, none of the compounds tested from any category formed methoxylamine conjugate, a reaction with putative aldehyde moiety formed via acyl migration. These results, highly favor the nucleophilic displacement as a cause of the reactivity rather than the acyl migration via aldehyde formation. The workflow presented could also be applied in the discovery setting to triage new chemical entities of interest.

Keywords: UGT, acyl glucuronides, glucuronidation, reactivity, stability, bioactivation, thioethyl glutathione, methoxylamine.

1. INTRODUCTION

Glucuronidation is the conjugation of glucuronic acid to various functional groups, catalyzed by UDP-glucuronosyltransferases (UGTs) in the presence of activated co-substrate, uridine diphosphoglucuronic acid (UDPGA) [1, 2]. Glucuronides, the products of glucuronidation, are formed at nucleophilic functional groups such as aliphatic hydroxyl/phenolic groups, thiol, amino groups, as well carboxylic acid functionalities [3, 4]. N-carbamoyl glucuronides, glucuronides formed at carbon and selenium atoms, discrete and linked di-glucuronides are examples of uncommon glucuronides [5]. Acyl glucuronides are formed when carboxylic acid moieties are present or eventually unmasked (i.e. oxidatively or hydrolytically) in endogenous and exogenous molecules are metabolized by UGTs. Bilirubin mono- and di-glucuronides are examples of acyl glucuronides of endogenous molecules [6]. Non-steroidal anti-inflammatory drugs with acyl groups are a class of compounds that are metabolized via biotransformation to acyl glucuronides [7].

While glucuronides are generally perceived as ‘excreted end-products of detoxification reactions,’ acyl glucuronides of xenobiotics have gained wide interest with respect to biochemical reactivity, hepatic disposition, and enterohepatic circulation of the aglycone, i.e. formation of acyl glucuronides in the liver, excretion into bile and reabsorption/hydrolysis via intestine [8-14]. It has been hypothesized

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that 1-β acyl glucuronides or their thio-acyl-glutathione drug esters [15, 16] can be easily transacylated by nucleophilic groups of proteins. In addition, 1-β acyl glucuronides are believed to form protein adducts via glycation, a pathway that is initiated by acyl migration to the 2-, 3-, or 4- β isomers, followed by glycation via Amadori rearrangement. Specifically, acyl glucuronides of non-steroidal anti-inflammatory drugs undergo active uptake in the gut, followed by intracellular enzymatic and non-enzymatic hydrolysis to release the respective parent drugs. The resultant localized high concentrations of the aglycone are thought to be the cause of damaging effects [17]. In addition, zomepirac was withdrawn from the market in 1985 due to a reported number of damaging effects ranging from anaphylactic reactions to patient deaths [18]. Consequently, high scrutiny of non-steroid anti-inflammatory drugs has led to a common characterization of other carboxylic acid containing compounds as “safe,” “warning,” or “withdrawn” within the drug metabolism and translational medicine community. These categories and the unpredicted clinical toxicity have led to major developability concerns for clinical candidates containing free or masked carboxylic acid moieties, which could form acyl glucuronides.

Historically, researchers have proposed numerous conditions and methodologies to evaluate the reactivity and preclinical toxicity of carboxylic acid containing drugs, because of their corresponding acyl glucuronides. These reports however typically only focus on one aspect, often metabolite stability differs for each category [19] or in vitro or in vivo metabolite conjugation of a withdrawn drug [20, 21]. The stability of acyl glucuronides has been studied in buffers, plasma, or human serum albumin [22, 23]. Additionally, reactivity with peptides [24] or evaluation of the disappearance of the anomeric resonance of 1-β-acyl glucuronide by NMR [25] are acknowledged as reasonable surrogates for biochemical reactivity. Nevertheless, due to a poor understanding on the reactive species that drives toxicity and the lack of preclinical assays to predict clinical observations, risk assessment of acyl glucuronides remains a challenge.

In the present investigation, we aimed to study the bioactivation risk of acyl glucuronides of more than 10 drugs (Fig. 1) by their acyl migration potential, measured by the number of peaks detected via LC-MS separation, coupled with in vitro trapping studies with reduced glutathione or methoxylamine. The trapping studies were designed to sequester the putative reactive acyl glucuronide itself and the ring opened aldehyde form of the substituted glucuronide, representative of the known transacylation and glycation pathways, respectively. We eliminated the need for chemical synthesis of an acyl glucuronide by utilizing lyophilized extracts from an in vitro incubation as a surrogate. We evaluated half-lives of the biosynthesized acyl glucuronides as well as authentic reference standards of acyl glucuronides under various conditions, referred to as ‘stability’ in many literature reports to date. While UGTs 1A1 and 2B7 have been widely studied, comprehensive

Fig. (1). Structures of the parent drugs, which form acyl glucuronides, utilized in the present investigation.
reaction phenotyping information on drugs containing carboxylic acids is scarce. Thus, we explored acyl glucuronidation of the commercial drugs with recombinant UGT Supersomes® to address this gap in the literature.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

All of the aglycones were obtained from Novartis Institutes for Biomedical Research compound bank. All of the acyl glucuronide standards were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Human liver microsomes (20 mg/mL, donor pool of 50, mixed gender) and UGT Supersomes® at 5 mg/mL protein concentration (UGT1A1, UGT1A3, UGT1A6, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17) were obtained from BD Biosciences (Franklin Lakes, NJ now Corning). UDPGA and methoxylamine were purchased from Sigma-Aldrich (St.Louis, MO). Reduced glutathione was purchased from Acros Organics (Fairlawn, NJ). Other solvents and reagents were MS grade and were purchased from Fisher Scientific (Fairlawn, NJ).

2.2. In vitro Stability of Acyl Glucuronides Standards

Acyl glucuronide standards (20 µM final concentrations) were incubated in 0.1 M KH$_2$PO$_4$ buffer (pH 7.4) at 37°C. Time points were taken at 0, 1, 2, and 4 hours and quenched with equal amount of acetonitrile containing 0, 0.1, or 1% formic acid. The samples were analyzed by LC-MS$^®$ analyses without any further treatment.

2.3. In vitro Biosynthesis of Acyl Glucuronides

Glucuronidation experiments were conducted by modification of previously reported protocols [26-28]. In short, the incubations (1.5 mL) were conducted in 2 mL 96-deepwell-plates (Analytical Sales and Products, Inc., Pompton Plains, NJ), at 37°C in a shaking water bath. The incubations contained human liver microsomes at a protein concentration of 1 mg/mL or human UGT isoforms at a protein concentration of 0.2 mg/mL in 0.1 M KH$_2$PO$_4$ buffer (pH 7.4), substrates (20 µM), alamethicin (50 µg/mL), MgCl$_2$ (5 mM), and D-saccharolactone (1 mg/mL). Each reaction was started by the addition of UDPGA (3.2 mg/mL). After 2 hours of incubation, the reaction was quenched with 1.5 mL of acetonitrile and centrifuged for 5 minutes at 4630 x g. The resulting supernatants were transferred to clean 15 mL conical polypropylene tubes and stored at -80°C.

2.4. In vitro Stability of Biosynthesized Acyl Glucuronides

The biosynthesized acyl glucuronides were lyophilized using Labconco lyophilizer (Kansas City, MO). The residues containing the acyl glucuronides were re-suspended in 1.5 mL of 0.1 M KH$_2$PO$_4$ buffer (pH 7.4) and 300 µL were immediately transferred to another clean vial containing 300 µL of acetonitrile with 0 or 1% formic acid to make the 0 hour time point. Subsequent time points at 1, 2, and 4 hours were processed in the same way as the 0 hour time point. All time points were analyzed by Orbitrap Elite MS$^®$ instrument (Thermo Fisher Scientific, Waltham, MA) without any further treatment.

2.5. In vitro Reactivity of Acyl Glucuronides

Acyl glucuronide standards (10 µM) were incubated in 0.1 M KH$_2$PO$_4$ buffer (pH 7.4) at 37°C with either reduced glutathione (5 mM) or methoxylamine (1 mM) as trapping agents. The reaction was quenched at 0 hour (control) and 2 hour as previously described.

2.6. LC-MS$^®$ Method for Metabolite Identification and Analysis

Samples were analyzed using a Orbitrap Elite mass spectrometer capable of MS$^®$ scanning and accurate mass measurement interfaced with a Dionex Ultimate 3000 (Thermo Fisher Scientific) HPLC pump and CTC PAL autosampler (Leap Technologies, Carboro, NC). This was achieved by modification of previously published methods [29, 30]. In short, the analytes (injected as either 20 µL or 60 µL aliquots) were separated on a Waters Symmetry C18 analytical column (5 µm, 2.1 x 150 mm, Milford, MA) with a 35 minute gradient elution method. Mobile phase A consisted of 10 mM ammonium formate buffer in MS grade water. Mobile phase B consisted of MS grade acetonitrile. The sample aliquots were eluted with a flow rate of 0.25 mL/min with 5% B over 5 minutes. Thereafter, the percentage of mobile phase B was gradually increased to 90% B over 25 minutes. Following the elution of the aglycone and acyl glucuronide, the column was returned to 10% B over 1 minute, where it was held for 3 minutes before the next injection. The analysis was carried out in positive and/or negative electrospray ionization with a source voltage of +4.00 or -3.5 kV, respectively. Relevant tune parameters also included a capillary temperature of 300°C, sheath gas flow rate of 30 mL/minute, source current of 100 µA, and S-lens RF level of 68%. Fourier transformation enabled accurate mass measurements were carried out at a mass resolution of 30,000. Data dependent scan after collision induced dissociation was carried out in the ion trap at normalized collision energy of 35. Activation Q was set at 0.25 and activation time was 10 milliseconds.

3. RESULTS

3.1. Stability

All of the aglycones used in the study formed the respective acyl glucuronide metabolite in human liver microsomes. Acyl glucuronides of ibufenac and fenclofenac were excluded from half-life calculations due to their spontaneous rearrangement and difficulty in detection of the main isofor with the current LC-MS$^®$ method. In general, the half-lives were consistent with reported values at 1% of formic acid among the various conditions studied in this investigation. Logarithmic half-lives of the biosynthesized acyl glucuronides were in alignment with those obtained with authentic standards. Fig. (2) shows trend analysis of the observed half-lives in each assay condition. Our results also demonstrated substantial differences between drugs categorized as “withdrawn” and “warning” in comparison to the drugs categorized as “safe.” Overall, half-lives of ≥ 10 hours as evident
for the drugs from the “safe” category indicated that the acyl glucuronides were not reactive. Interestingly, as observed during LC-MS analysis, all the acyl glucuronides from the “safe” category showed a single chromatographic peak up to 4 hours in contrast to the acyl glucuronides from the “warning” and “withdrawn” categories. This property may be useful as another marker of a glucuronide’s reactivity.

3.2. Reactivity

As shown in Fig. (3), one pathway in which the toxicity of acyl glucuronides is postulated to occur is via nucleophilic displacement, which involves the nucleophilic attack by a protein’s functional group (−NH₂, −SH, −OH) to the carbonyl carbon atom. The other pathway is via imine formation during intramolecular rearrangement of the substituted hexuronic acid (glucuronide functionality), where the aldehyde reacts with amino groups of proteins. We supplemented our incubations with reduced glutathione and/or methoxylamine as nucleophilic agents to mimic the proteins (Fig. 3). While none of the acyl glucuronides from any categories (“withdrawn”, “warning” or “safe”) formed methoxylamine conjugates, all of the acyl glucuronides from the “withdrawn” and many from the “warning” category formed glutathione adducts. The acyl glucuronides from the “safe” category did not form any glutathione adducts. Glutathione adducts were also not observed on the parent drugs containing the carboxylic acid (data not shown). Thus, the glutathione trapping assay may be a good surrogate to investigate the reactivity of the acyl glucuronides, as a complimentary method to the stability assay. This data is summarized in Table 1.

3.3. Reaction Phenotyping

It has been hypothesized that UGT1A1 and UGT2B7 are important enzymes that aid glucuronidation of carboxylic acids. Reaction phenotyping of the drugs in the present investigation revealed contributions from enzymes such as UGT1A3, UGT2B15, and UGT2B17 toward glucuronidation of carboxylic acid drugs. Normalization of acyl glucuronide relative peak areas to UGT protein content as per the quantitative proteomics approach by Smith and co-workers did not remarkably alter the results or change the interpretation from these experiments [31]. UGT1A7, UGT1A10, and UGT2B10 did not metabolize any of the studied drugs. UGT1A4, which is known to primarily metabolize amines, was excluded from the present investigation due to substrate specificity. The results are shown in Fig. (4). As a result, the assay can be easily carried out in a 96-well format to screen multiple analogs during lead optimization phases of a program. Interestingly, none of the recombinant UGT enzymes studied in the present investigation were found to metabolize ibufenac to its corresponding glucuronide.
**Table 1.** The number of peaks observed for each acyl glucuronide post reconstitution with acetonitrile containing 1% formic acid, and presence of observed adducts upon reaction with reduced glutathione or methoxylamine. The acyl glucuronides tested herein, were either authentic reference standards obtained from commercial sources. YES indicates that an adduct could be identified and characterized by high resolution LC-MS\(^\text{b}\), whereas ‘-’ indicates otherwise. ‘n/a’ indicates that the assay could not be conducted due lack of availability or purity of the reference standard.

| Acyl Glucuronide (Reference Standard >95% Purity) | Number of Peaks Observed for the Acyl Glucuronide | Conjugate Identified with Reduced Glutathione | Conjugate Identified with Methoxylamine |
|-------------------------------------------------|--------------------------------------------------|-----------------------------------------------|----------------------------------------|
| **SAFE**                                        |                                                  |                                               |                                        |
| Gemfibrozil acyl glucuronide                    | 1                                                | -                                             | -                                      |
| Montelukast acyl glucuronide                    | 1                                                | -                                             | -                                      |
| Telmisartan acyl glucuronide                    | 1                                                | -                                             | -                                      |
| Repaglinide acyl glucuronide                    | 1                                                | n/a                                           | n/a                                    |
| **WARNING**                                     |                                                  |                                               |                                        |
| Diclofenac acyl glucuronide                     | 3                                                | YES                                           | -                                      |
| Ibuprofen acyl glucuronide                      | 4                                                | YES                                           | -                                      |
| R-Naproxen acyl glucuronide                     | 3                                                | -                                             | -                                      |
| Furosemide acyl glucuronide                     | 3                                                | -                                             | -                                      |
| Indomethacin acyl glucuronide                   | 4                                                | n/a                                           | n/a                                    |
| **WITHDRAWN**                                   |                                                  |                                               |                                        |
| Zomepirac acyl glucuronide                      | 4                                                | YES                                           | -                                      |
| Fenclofenac acyl glucuronide                    | n/a                                              | YES                                           | -                                      |
| Ibufenac acyl glucuronide                       | n/a                                              | YES                                           | -                                      |
4. DISCUSSION

4.1. Rearrangement and Reactivity: Anomerization, Isomerization, Transacylation and Glycation

The acyl glucuronide biosynthesis assay was in agreement with the work of Jinno and coworkers [19], and could be easily applied in a drug discovery setting, where the authentic standards of acyl glucuronides are often unavailable. Upon formation, the acyl 1-β-glucuronides undergo acyl migration to the 2-, 3-, or 4-β isomers. The 2-, 3-, and 4-β forms may undergo reversible anomerization to their corresponding 2-, 3-, 4-α-isomers, respectively. It is noteworthy to mention that the α-isomers cannot be cleaved by tissue- or blood-β-glucuronidases. Anomerization has been documented to be predominant at lower pH values and is required to proceed through ring opening of the glucuronolactone. An open hexuronic acid form of the glucuronide, which includes a free aldehyde group, serves as an intermediate during anomerization and is assumed to be the critical reactive intermediate in the formation of protein adducts. The reaction with nucleophilic amino groups of a protein, followed by loss of water leads to the formation of Schiff’s base type protein adducts. These protein adducts undergo molecular rearrangement driven by the formation of a stable 1-amino-1-deoxy-2-ketose form. This last step of the glycation bioactivation pathway is known as Amadori rearrangement. Due to the inherent nature of this biochemical pathway, Amadori rearrangement can only proceed subsequent to formation of 3- and 4- positional isomers of the glucuronide metabolites. The 1- isomer cannot form the open hexuronic acid intermediate and 2- positional isomers cannot undergo Amadori rearrangement [32]. None of the acyl glucuronides tested in the present investigation reacted with methoxylamine to form the expected methoxylamine conjugates. The data strongly suggest that the open hexuronic acid form of the glucuronide may not be as readily formed under incubation conditions at physiological pH, thus pointing toward anomerization as a lesser feasible of the two bioactivation pathways. Acyl glucuronides have been documented to undergo bioactivation, via transacylation leading to the direct displacement of the glucuronic acid moiety. Transacylation is shown to be promoted at higher pH values and may possibly transpire with any and all reactive positional isomers of acyl glucuronides. This transacylation reaction is thought to occur with proteins or with glutathione; the latter leads to the formation of thio-acyl glutathione conjugates which may eventually form protein adducts [15, 16]. The Grillo laboratory demonstrated that thio-acyl glutathione conjugate of diclofenac was more reactive toward N-acetyl cysteine than the corresponding acyl glucuronide [16]. This work included in-depth mechanistic assessments of a few compounds. However, in the present investigation, the transacylation concept via glutathione adduct formation was observed for drugs in the “warning” and “withdrawn” categories (Supplemental Fig. 1). None of the drugs in the “safe” category formed any adducts. This assay highlights a simple in vitro screen to rank compounds in a series for their reactivity in a drug discovery setting, without the need for specialized or labor intensive studies, hepatocytes or in vivo bile duct cannulated surgical models. Interestingly, the total acyl glucuronide formed was not suggestive of the reactivity or the parent drug’s categorization.
4.2. Reaction Phenotyping

Iwamura and coworkers have tabulated a list of UGTs responsible for acyl drugs from various sources [33]. However, reaction phenotyping information for all the drugs in the present investigation was unavailable. We conducted these studies in an effort to comprehensively evaluate the contributions of UGTs. While it is hypothesized that UGT1A1 and UGT2B7 are important enzymes that aid glucuronidation of carboxylic acids, our results indicate that UGT1A3, UGT2B15, and UGT2B17 among other UGTs are also noteworthy from a perspective of glucuronidation of carboxylic acid containing drugs. The latter two enzymes have gained wide attention due to their ontogeny and genetic polymorphisms [34, 35]. It cannot be ruled out that ibufenac may be a substrate for UGT enzymes from 3A or 8 families, which are only known to form N-acetylg glucosamine, xylose or glucose conjugates. It also possible that ibufenac is metabolized by hetero- dimers or tetramers of UGTs, which may be present in microsomes but not Supersomes®. Why ibufenac failed the reaction phenotyping assay is unclear.

4.3. Acyl Glucuronide Considerations and Challenges

The ultimate utility of this investigation was to accurately estimate acyl glucuronide reactivity, the subsequent translation to the proportion of acyl-glucuronide formed in humans, and correlate with retrospective evidence of observed toxicity. To place that ideology into practice, there are several practical limitations in the present day. Despite remarkable advances in mass spectrometric soft spot identification technologies [29, 36], glucuronides still present many analytical challenges. Quantification of acyl glucuronides cannot be performed without an authentic and pure reference standard because ionization of the parent and the acyl glucuronide unpredictably vary even under the exact same LC-MS conditions [37, 38]. Thus, estimation of reaction kinetics is seldom possible in discovery and early development [39]. Accurate and precise in vitro-in vivo correlation of amounts of acyl glucuronides is limited because of inestimable phenomena such as enterohepatic circulation, extrahepatic contribution to glucuronidation, differences in enzyme expression levels, competing metabolic pathways, etc. Single species scaling, relative activity factors, and fraction-metabolized methods aid the translatability of preclinical data to the clinics [40-42]. However, predicting clinical pharmacokinetics remains a challenge.

We aimed to place the bioactivation potential and reactivity of acyl glucuronides in an appropriate context in drug discovery and early development. To this effect, we present a guidance work stream to investigate acyl glucuronides (Fig. 5), taking the reactivity and stability into account. A rapid derivatization with 55% hydroxyl amine solution converts acyl glucuronides to their corresponding hydroxamic acids [43] and can serve as a quick confirmatory test. This is useful in case the compounds of interest have multiple groups which may undergo conjugation reactions. Stability assays, either via biosynthesis or in rare cases where reference standards of glucuronides are available, set the stage for inferring half-lives [22, 23] and number of peaks in LC-MS analysis as shown in the present investigation (Supplemental Fig. 2).

Fig. (5). Risk assessment of acyl glucuronides in discovery.
Reaction with reduced glutathione provides another dimension to the reactivity component, as described in this analysis. Alternatively, the biochemical reactivity may be assessed with peptides, although these nucleophiles have been historically demonstrated for a small subset of compounds [24]. Where pure reference standards of acyl glucuronides are available, NMR based methodologies to detect the disappearance of the anomic resonance of 1-β-acyl glucuronide [25] or immunostimulation in human peripheral blood monocytes may also serve as a useful surrogate markers of reactivity [44]. In silico approaches such as the partial least squares model proposed by Potter and coworkers [45] can be broadened and qualified to support medicinal chemistry intervention strategies and synthesis of next generation analogs. Another aspect worthy of consideration is that observation of an acyl glucuronide as a ‘major’ metabolic pathway should not preclude progression of a new chemical entity into further development. For example, despite 50% of valproic acid being metabolized to its corresponding glucuronide in humans, by multiple UGTs, namely UGT1A4, UGT1A8, UGT1A10, and UGT2B7 [27], the metabolic pathways responsible for the hepatotoxicity of valproic acid are identified as mitochondrial oxidation and oxidative stress [46, 47]. Thus, as seen with valproic acid, acyl glucuronidation can be a protective pathway. Finally, the information obtained from various assays needs to be put in to proper context in order to assess the risks and benefits of any given clinical drug candidate.

Due to the potential developability concerns around drugs that form acyl glucuronides, a wide array of techniques have been reported over the years. While these techniques range from in silico measurements to detailed in vitro and in vivo characterization, none of the techniques is predictive of human pharmacokinetic/toxicokinetic behavior or toxicological implications. Even in a study investigating formation of acyl glucuronides and thio-acyl CoA conjugates with radiolabeled aglycones, covalent binding to microsomal proteins could not be attributed via acyl glucuronidation pathway [48]. A separate study also showed that thio-acyl conjugates solely were not indicative of drug induced liver injury [49]. Fig. (5) presents a comprehensive risk mitigation strategy by applying a combination of available assays. In early development, the drug candidate undergoes extensive characterization and risk management as per the guidelines issued by the health authorities. However, in drug discovery research, the outlined assays will help rank order compounds within a chemical series, and if needed, devise chemical intervention strategies to minimize the risk. For example, chemistry teams can modulate physico-chemical properties to redirect metabolism or make chemical modifications to the core including application of bio-isosteres. Such an approach may be employed in discovery for evaluation of acyl glucuronide reactivity and subsequent risk assessment of potential lead candidates.

In summary, we show distinctive features of acyl glucuronides of most drugs from the “safe” category in comparison with those the “warning and/or withdrawn” category. Half-lives, number of chromatographic peaks in LC-MS², reactivity with glutathione can be valuable markers in assessing risks of acyl glucuronides. We have contrasted our findings with those in the literature and shown that a representative decision making guidance work stream (Fig. 5) may be developed to triage new chemical entities. Although, the evidence for clinical translation of these assays is lacking, these assays serve as a valuable tool, especially, in drug discovery. A decision making system, as presented herein, serves as an invaluable means to accurately time the comprehensive risk assessment.

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