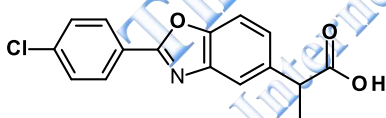


## REACTIVE METABOLITES FROM CARBOXYLIC ACIDS

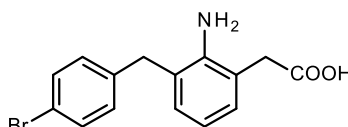
1. Mechanisms
2. Selected evidence from the literature
3. Some tentative conclusions regarding safety of carboxylic acids in drug design
4. Suggested tentative guidance

The carboxylic acid (CA) functional group is quite common among therapeutic drugs: around 14% of all drugs in GVK Bio's and DrugBank's drug databases in 2018 were CAs.

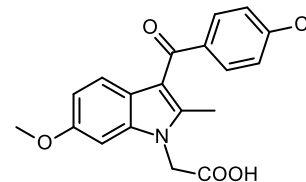
Many NSAIDs (COX-1/COX-2 inhibitors), nearly all of which are CAs, have been associated with liver injuries or other related adverse effects. A total of about twenty NSAIDs have been withdrawn from the market. These include, e.g. [benoxaprofen](#), flunoxaprofen, [bromfenac](#), [clometacin](#), [fenclozic acid](#), ibufenac, fenclofenac, [zomepirac](#), and, as late as 2007, [lumiracoxib](#) (most structures shown below). The NSAID class of drugs also encompasses some non-CAs such as the acidic structures of [phenbutazones](#) and the [oxicams](#). All these structures invariably contain substituted benzene rings, in some cases also anilines, which are often combined with heteroaromatics. A brief overview of the available and of some withdrawn NSAIDs is found on the [LiverTox](#) site.



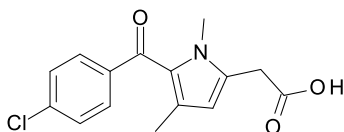
R=Cl benoxaprofen; R=F flunoxaprofen



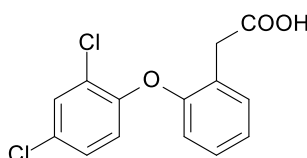
Bromfenac



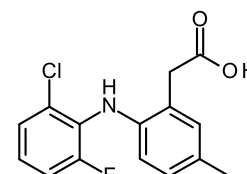
Clometacin



Zomepirac

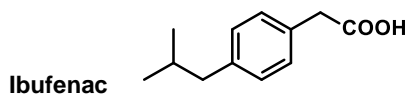


Fenclofenac

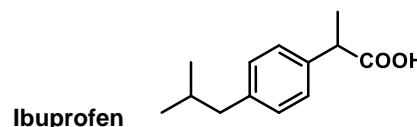


Lumiracoxib

Structures less compromised by halogen-substituted aromatics are found in the following two NSAIDs: ibufenac was withdrawn from the European market in 1968 while ibuprofen is still widely used and from a reactive metabolite (RM) perspective is considered to be among the safest NSAIDs (Smith *et al.* [Book cited](#) in Google 2010). This is an enigmatic example of a minor structural difference giving a huge safety effect that not until 2015 reached a plausible mechanistic explanation (more below).



Ibufenac



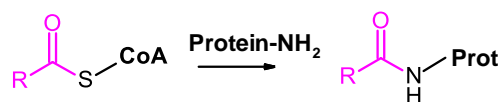
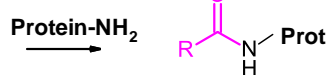
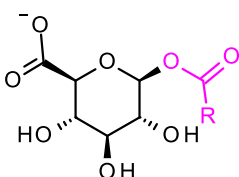
Ibuprofen

In the following sections some of the evidence for the involvement of the CA part of these compounds in causing the ADR problems is scrutinized. The discussion is then extended to other CA drugs with and without RM problems, and a balanced view on the RM prospects of the carboxylic acid group in medicinal chemistry will hopefully be presented. For a collection of references to reviews on the topic, visit [this](#) site.

## 1. Mechanisms

There are two major mechanisms by which CAs can *acylate* macromolecules (a process sometimes referred to as transacylation): 1) via acyl glucuronides (AG) acting as acylating agents (active ester) which transfer the drug to an amino group in a macromolecule, as shown below, and 2) via thioesters, from Coenzyme A (CoA), which are also reactive esters. In addition, there is 3) the *glycation* pathway described below wherein the drug together with the glucuronic acid moiety becomes attached to a protein.

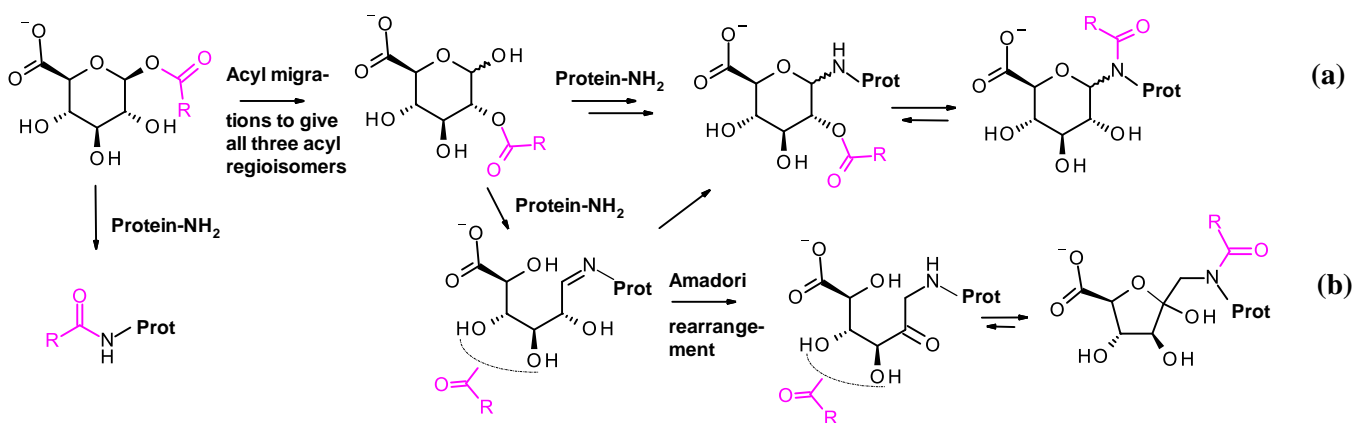
AGs are formed from glucuronosyl-UDP, catalyzed by uridyl glucuronosyltransferases, by nucleophilic ( $S_N2$ ) attack of the  $RCOO^-$  on the glycosidic carbon whereas a CoA thioester is formed by the thiol of CoA attacking an acyl-adenylate derivative. The intermediate acyl-adenylate is very reactive and could in principle act as a RM but might not be as accessible to various proteins as the acyl CoA thioesters are.



Reaction 1. Acyl transfer via glucuronide

Reaction 2. Acyl transfer from CoA derivative

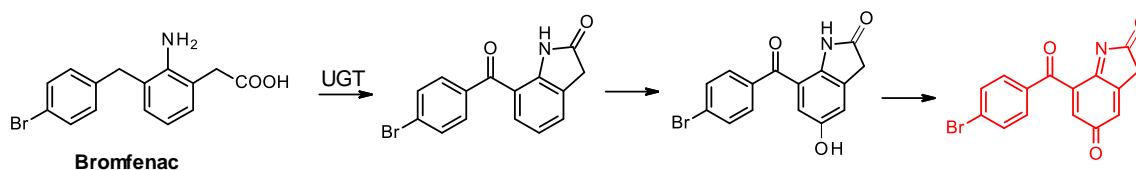
It has been shown that a CA can also be attached to macromolecules via initial rearrangement (O-acyl migration) to expose the sugar aldehyde/hemiacetal, which can lead to imine formation with an amino group in a protein followed by more acyl migrations, possibly ending in a stable amide (glycation path **a**). The initial imine formation may be followed by a prototropic rearrangement (Amadori rearrangement in sugars) to give the products illustrated below (glycation path **b**) where the drug acyl group eventually migrates to form a stable amide including the three components: drug, protein, and glucuronic acid. [This is also how proteins can become non-enzymatically and irreversibly glycated by (excess) sugars, mainly glucose, thus contributing to the physiological problems in diabetic patients.]



Such adduct structures initiated by imine formation have been identified from diclofenac on human albumin (HSA), [Hammond et al. \(2014\)](#) and Nygaard [Monrad et al. \(2014\)](#), who studied in great detail the adducts from the pair ibuprofen/ibufenac, 4-bromobenzoic acid (BBA), and other carboxylic acids (see below under section 2. Selected Evidence..).

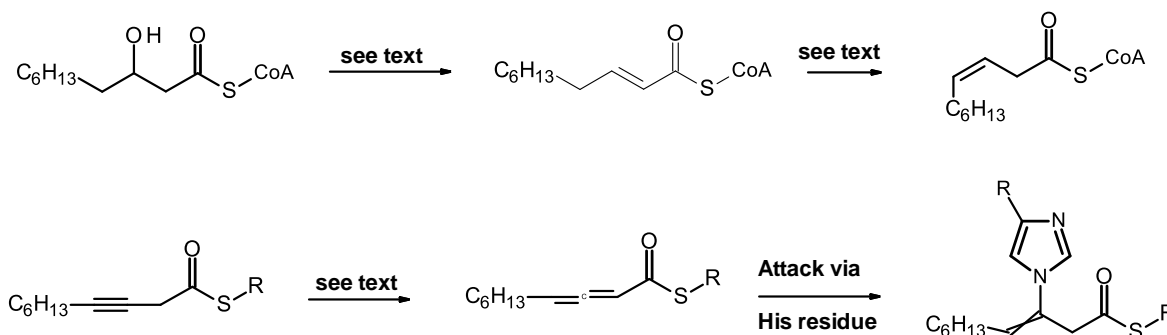
Earlier, [Ding et al. \(1993\)](#) reported that tolmetin AG (structure below) reacted with serum albumin in vitro to give several adducts (identified by MS on tryptic digests) containing *both the tolmetin and the glucuronic acid moieties*. This implies that the tolmetin AG could have acylated the albumin protein as well as reacted via other routes involving the rearranged AG, as in the scheme above [more fully discussed by [Darnell & Weidolf \(2013\)](#) and by [Hammond et al. \(2014\)](#), specifically regarding diclofenac; the latter authors cite several earlier studies on diclofenac AG, where the adduct with proteins retained the glucuronic acid moiety]. [Smith et al. \(1990\)](#) detected acid-labile adducts derived from zomepirac [ $^{14}\text{C}$ ]-AG with albumin in vitro, which might indicate adducts to the sugar aldehyde of the rearranged AG.

The fate of an AG might also be facilitated conversion of the carboxylic acid into a neutral lactam by an intramolecular reaction, such as in the case of bromfenac (withdrawn in 1998). Incubating the compound with UDPG and UGT leads only to formation of the lactam shown below, [Driscoll et al. CRT2018](#). This neutral oxindole then undergoes the expected CYP oxidation to a phenol eventually giving rise to a reactive quinoneimine.



**CoA conjugates.** Regarding RMs from CAs, most focus has been on the AG conjugates. However, in addition to serving as activated esters of drugs, the CoA thioesters can become substrates for enzymes normally active in fatty acid metabolism. This has become more in focus during the last couple of decades but has a longer history.

Around 1970 Konrad Bloch's group studied the *E. Coli* enzyme  $\beta$ -hydroxy-decanoyl thioester dehydrase that catalyzes the conversion of 3-hydroxydecanoyl-thioester to the corresponding 2-enoate, which by the same enzyme is then isomerized to the *cis*-3-enoate (Scheme below). The research group accidentally found that 3-decynoyl thioesters inhibit both reactions irreversibly: the mechanism, they found, was that the enzyme isomerizes the acetylenic group to a reactive allenic intermediate, as shown below, which is attacked by a His imidazole in the enzyme's active



site leading to irreversible inhibition.

This now classical example was summarized by Bloch in a 1986 [essay](#) termed “The beginnings of enzyme suicide”. The pioneering discovery led to coining of the term “suicide inactivation”, which has since mostly been referred to as “mechanism-based inhibition”. The concept has led to

new drug designs and nicely explained many known irreversible enzyme inhibitions, in particular of the cytochrome P450 enzymes often mentioned within SpotRM. As applied to CoA thioesters of CAs the concept might be labelled *lethal synthesis* instead of RM formation (*cf.* incorporation of fluoroacetic acid into (-)-*erythro*-2-fluorocitric acid, separate [monograph](#) and [Wiki](#)).

One early example where CoA derivatives are involved in DILI is provided by [valproic acid](#) in which the CoA conjugate is further oxidized in the alkyl chains (p. 5). Another example is given below by a “MRL-A” compound (p.6). The CoA derivative can also here be described as a “suicide inactivator” of a specific enzyme.

## 2. Selected evidence from the literature

The question of the role(s) of AGs for idiosyncratic drug reaction is well summarized by [Hammond et al.](#) (2014): “From the earliest identifications of AG as unstable and protein-reactive conjugates these commonplace metabolites have been linked persistently, and at times almost generically, but always somewhat uncertainly, with the varied adverse reactions of carboxylate drugs (Regan et al., 2010; Sawamura et al., 2010). This hypothetical linkage of protein adduction and toxicity has been particularly enduring but no less contentious in the case of hypersensitivity reactions to nonsteroidal anti-inflammatory drugs (NSAIDs), such as diclofenac.” See the [diclofenac](#) monograph for more details.

A 2018 commentary, “Safety Assessment of Acyl Glucuronides – A Simplified Paradigm” by [Smith et al.](#), provides further safety aspects to the subject from a MIST (Metabolites in Safety Testing) perspective. See also the [FDA guidelines](#) on MIST from 2016, which mention AGs as “toxic metabolites” (US Food and Drug Administration, 2016) despite lack of solid evidence for *in vivo* toxicity attributable to an acyl glucuronide (see also extensive discussion in a review on RM alerts by [Kalgutkar 2019](#) and critical views by Jack Utrecht on p.6).

Model experiments using AGs and proteins, mostly albumin, have confirmed that proteins can be modified via AGs. However, the importance of this pathway for antigen formation does not appear clearly established, as the above citation indicates. This is in spite of the fact that glucuronidation is often a dominating route of CA metabolism. The other mechanism for detrimental activation of CAs, i.e., via thioester formation with CoA, generates more reactive acyl derivatives and would seem more plausible from a purely chemical reactivity perspective ([Olsen et al.](#) 2002). During the period from around 2000 till now the evidence, as outlined below, has grown stronger for this view; a 2013 review by [Darnell & Weidolf](#) highlights this discrepancy and details ways of studying CoA esters of xenobiotic CAs. As discussed earlier, CoA thioesters of CAs, in addition to serving as reactive acylating agents of proteins, likely also interfere with enzymes that affect  $\beta$ -oxidation of fatty acids in a way that might be labelled *lethal synthesis*.

Nygaard [Monrad et al.](#) (2014) reported a surprisingly high rate of BBA glycation of HSA after 16 h (57% of HSA had reacted) compared with ibuprofen, ~ 2%. Ibuprofen reacted with HSA mainly, to ~2/3, via acyl transfer while this pathway was negligible for BBA. This is in contrast with the general view of AGs of benzoic acids drugs as being more stable than those of alkyl CAs (see next paragraph and references in [Iwamura et al. DMPK2017](#)). However, probenecid-AG, which has a 4-sulfonyl group, is more labile than other benzoic acid-AGs.

Reactivity of AGs varies considerably between alkyl CAs, much depending on the alpha substitution. [Zhong et al. \(DMD2015\)](#) measured the rates of acyl migration/isomerization of 23 AGs as a means of establishing a method for assessing potential development risks of drug

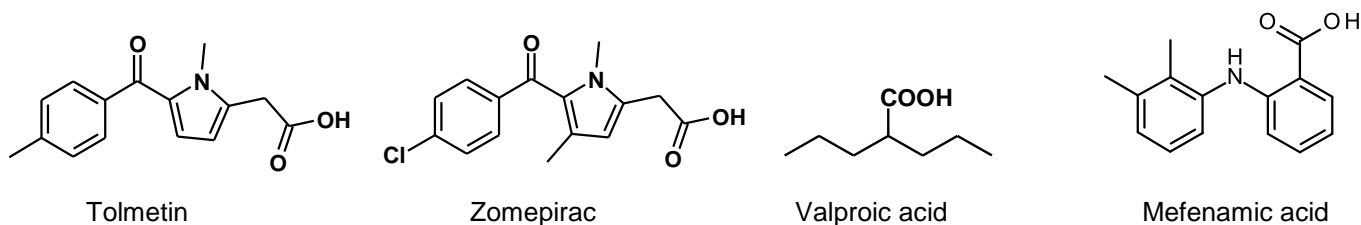
candidates. It is quite clear that arylacetic acids are more labile in this respect than arylpropionic acids (aryl  $\alpha$ -methylacetic acids). The stability is in good agreement with hydrolytic stability.

There have been attempts to correlate reactivity of AGs with the “idiosyncratic drug toxicity risk” of carboxylic acid drugs, for example by Sawamura *et al.* (DMD2010, 19 drugs evaluated), by Lassila *et al.* (CRT2015; 13 drugs), and by Iwamura *et al.* (DMPK2017; attempt at assessment of methods). The wide variety of drug structures included in these studies makes it somewhat difficult to concur with the authors of the first-mentioned paper conclusions about predictability, even when the arylacetic acids, in contrast to 2-arylpropionic acids, do come out as the riskiest ones.

Similar conclusions were reached by Lassila *et al.* (CRT2015) based on studies of 13 drugs: “On the basis of the results, the short relative half-life of the acyl glucuronide (high acyl migration rate), high daily dose and detection of acyl CoA conjugates, or further metabolites derived from acyl CoA together seem to indicate that carboxylic acid-containing drugs have a higher probability to cause drug-induced liver injury (DILI).”

Hammond *et al.* (JPET2014) reported detailed studies on human serum albumin (HSA) adduct formation with diclofenac AG. In short, targeted mass spectrometric analyses of HSA isolated from six diclofenac patients to characterize drug-derived structures led to the conclusion that N-acylated albumin is formed while it was not possible to identify glycation products. However, such products were identified from the *in vitro* studies. The formation of albumin N-acylated with diclofenac had no consequences regarding hypersensitivity of the six patients investigated.

Valproic acid is a high-dose drug that has been shown to form a fairly stable AG that has only a slight tendency to react with amino groups (see review by Bailey&Dickinson 2003). This drug causes ADRs and the formation of an acyl-CoA thioester as the initiating event is regarded as proven (Chang & Abbott, 2006). Further support for the less noted pathway of thioester formation was provided by Olsen *et al.* (2007) who in several papers discussed the covalent binding of tolmetin, zomepirac, and 2-phenylpropionic acid to proteins. They concluded that the CoA thioester pathway, in addition to important CYP-mediated oxidative ones, should be regarded as an important mode of bioactivation of these carboxylic acids to RMs.



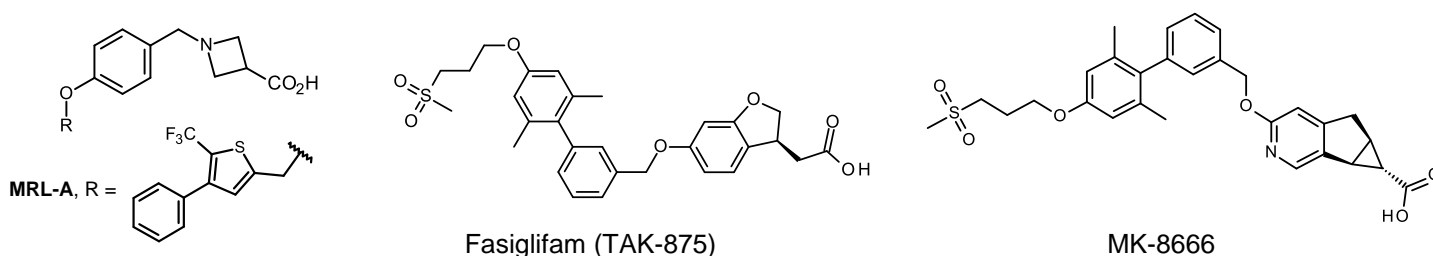
There are also studies of reactivities of mefenamic acid, its AG, CoA thioester, and acyl-adenylate to thiol and amino nucleophiles, Horng & Benet (2013) and Grillo *et al.* (2012). The oxidative RM-generating metabolism of mefenamic acid was studied by Venkataraman *et al.* and that of the close analogue meclofenamic acid by Schleiff *et al.* (see [this](#) monograph).

The striking difference between ibufenac and ibuprofen (structures on p. 1) with regard to safety was first addressed by Castillo and Smith (1995) in experiments that focused solely on AGs. On the basis of several types of covalent binding to plasma proteins in rhesus monkeys and *in vitro*, and on chemical stability, they concluded that the AG from ibufenac is 2-3 times as reactive as the one from ibuprofen (transacylation and glycation pathways summarized). This small difference in reactivity would not seem sufficient to explain the different safety profiles of these two NSAIDs. The conundrum can possibly be considered solved due, to a large part, to

AstraZeneca researchers who published a paper on the “*Significantly Different Covalent Binding of Oxidative Metabolites, Acyl Glucuronides, and S-Acyl CoA Conjugates Formed from Xenobiotic Carboxylic Acids in Human Liver Microsomes*” (Darnell *et al.* 2015). The authors describe a huge difference between ibufenac and ibuprofen regarding covalent binding via routes that must involve CoA thioester formation. The research group studied seven NSAIDs and points to the acyl-CoA and oxidative (of the aromatic parts) metabolic routes as being generally more important for RM formation than the AGs. Further analysis of CA activation was presented by Kalgutkar 2019, who devoted 10 percent of his broad review to CAs as RM alerts.

Another CA, MRL-A (below), a clinical candidate, was reported to inactivate rat long chain acyl-CoA-synthase 1 (ACSL1); details can be found in the monograph A-971432.

The GPR40 agonist fasiglifam was terminated in phase 3 studies in 2013 due to liver safety concerns. A tentative conclusion was that the AG conjugate played a major role in the toxicity while not excluding contributions from interference with fatty acid oxidation pathways and/or inhibition of bile acid transporters.



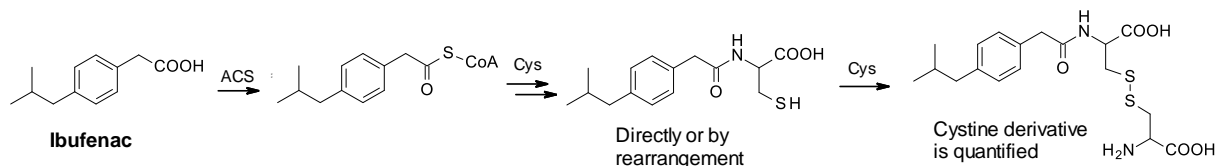
Another GPR40 agonist, MK-8666, was highlighted in early 2020 for exactly the same issues (CRT20); it was discontinued in phase 1 clinical trials due to liver safety concerns.

### 3. Some tentative conclusions regarding safety of carboxylic acids in drug design

The topic of reactive compounds (AGs and thioesters) from CAs has generated a large body of research reports, as summarised in recent reviews and papers comprising more than 140 papers (for example Skonberg *et al.* 2008; Bailey&Dickinson 2003; Darnell&Weidolf 2013 and CRT2015, Monrad *et al.* 2014, and Iwamura *et al.* 2016). It seems, however, that progress has been made and the conundrum has been largely resolved due to the increased insights regarding the significance of the acyl-CoA route to toxicity, especially through the results of Darnell *et al.* 2015. [Interestingly, it has been proposed that acylation of certain proteins with 3-hydroxy-3-methylglutaryl-CoA would be a normal mechanism for protein modification (Wagner *et al.* 2017)]. All this should feed new impetus into the drug discovery process making it possible to better assess carboxylic acids as drug candidates.

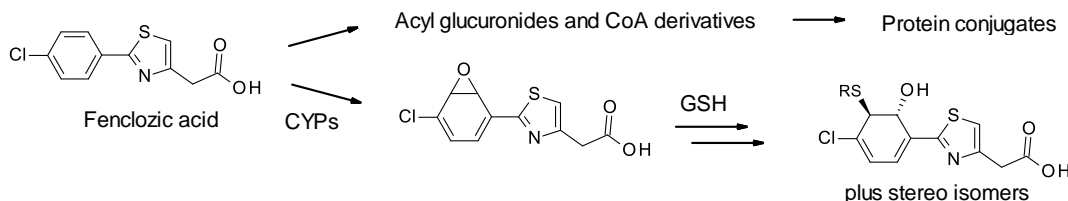
At the same time it has become obvious that the many acyl-CoA synthetases (ACSs) have widely different preferences. There are 26 distinct ACS genes (and five subfamilies) present in the human genome of which few are involved in xenobiotic metabolism (a summary is found in the 2013 paper by Darnell&Weidolf). As these authors conclude, determination of the relative contribution of each isoform to the overall metabolism of any given substrate *in vivo* will be very complicated. In addition, xenobiotic carboxylic acids are in many cases both substrates and competitive inhibitors of the enzymes.

Recently, a novel assay to monitor rates of CoA thioester formation was revealed by Japanese authors (Xen2022). In short, a CoA ester is allowed to react with cysteine ultimately resulting in a cysteine derivative that is quantified. Using [<sup>35</sup>S]Cys excellent quantification could be achieved. The procedure is illustrated below with ibufenac as an example.



The authors report rates of CoA ester formation for 17 CAs and could establish that ibufenac indeed has an outstanding high rate of CoA ester formation, about 12 times that of ibuprofen.

Many questions remain to be addressed at the various decision points where a preclinical compound might progress, for example and most importantly: from exactly where in the chemical structures are the found RM issues originating? Or are they a combination of contributions from different parts? Clearly, most of the structures of NSAIDs contain problematic aromatic substructures (*cf.* Claesson 2013), even being aromatic amines, *e.g.* diclofenac, and this fact ought to be better reflected when accounting for different RM mechanisms; see also the report by van Leeuwen *et al.* (2011) on the many differentials of NSAIDs toxicity. Boelsterlie 2003 did the same in a review on diclofenac hepatotoxicity. Another case in point was raised by Chen *et al.* (2006) who identified GSH adducts from the pyrrole part of zomepirac and tolmetin (see structures on p. 3) when these were incubated with liver microsomes (see monograph). Recent studies by Martin *et al.* (2014) on fenclozic acid (scheme below) underscore this point. From studies on *in vivo* metabolism the authors conclude that covalent binding to liver microsomes occurs as a result of initial epoxide formation on the benzene ring, as depicted below. This is despite the slow metabolic turnover of fenclozic acid. This drug was also included in the report by Darnell *et al.* 2015.



Further to this discussion, benoxaprofen (a withdrawn NSAID; see structure on p. 1) in a series of hepatotoxic and non-hepatotoxic drugs, has been assessed regarding covalent binding to proteins by Bauman *et al.* (2008) and by Obach *et al.* (2008). The first mentioned authors suggest that glucuronidation works in the direction of lowering covalent binding.

On the topic is also the mentioned 2013 review “Metabolism of Xenobiotic Carboxylic Acids: Focus on Coenzyme A Conjugation, Reactivity, and Interference with Lipid Metabolism” by Darnell & Weidolf and the paper by Weidolf’s group, Darnell *et al.* 2015.

Since long an authority on reactive metabolites, Jack Uetrecht, in 2020 summarized his critical views on why exaggerated focus on AG formation from CAs should come to an end:

*“The simple correlation that has been suggested between the reactivity of acyl glucuronides and the risk of IDRs is not at all clear. There is simply not convincing clinical evidence that the covalent binding associated with acyl glucuronide formation is responsible for serious IDRs. It is possible that acyl glucuronides can lead to IDRs but testing their reactivity during drug*

*development would lead to a large number of false positive and false negative results. Alternatively, acyl glucuronides may rarely or never cause IDRs. Yet, as stated earlier, many pharmaceutical companies routinely study the reactivity of acyl glucuronides of drug candidates that are carboxylic acids. In part, this is likely due to FDA guidance documents that imply that acyl glucuronides are a significant source of risk. It is possible to generate a large amount of data with high throughput in vitro assays; however, such data may do little to improve drug safety. What is needed is a better basic understanding of the in vivo immunological and other biological effects of drugs that lead to IDRs. There may be several mechanisms of IDRs. If that is the case there may never be a method that has a zero false negative predictive value, but if we understood some mechanisms well, it might be possible to have a method with a very low false positive rate. However, without a clear mechanistic understanding of the mechanisms of IDRs it is unlikely that any method will even have an acceptable false positive predictive value.”*

#### 4. Suggested tentative guidance

A summary of guidance gathered from the large number of reports published by specialists in the last 10-20 years may involve separate structural alert considerations for the following classes of CAs (see also perspectives in [CRT2018](#) by Claesson & Minidis; in [JMC2019](#) by Kalgutkar; and in [JMMC20](#) by Utrecht).

- *Arylacetic acids* (i.e., alpha-unsubstituted) – represent a definitive structural alert from both mechanistic aspects of AG and CoA thioester formations.
- *α-Alkyl arylacetic acids* (also referred to as α-arylpropionic acids when belonging to the NSAIDs group), although likely to be less hazardous than arylacetic acids are not totally harmless. Ibuprofen, for example, in contrast to several heterocyclic arylacetic acids gives rise to extensive covalent protein binding via the acyl CoA route ([Darnell et al. 2015](#)). General awareness and guidance by in vitro evaluations are recommended.
- *Aryloxyacetic acids*, which are shown to give highly reactive AGs have few relevant reference drugs and largely represent unprecedented territory (however, the hepatotoxic [tienilic acid](#) is an aryloxyacetic acid besides containing a thiophene). Another drug is [bendazac](#) (a 3-indazolyloxyacetic acid), withdrawn 1993 due to hepatotoxicity (Spain).
- Other alkyl (normal or branched) carboxylic acids deserve a vigilant view based on the cited valproic acid and fasilgifam/ MK8666 cases, and possibly on [amineptine](#).
- There seems to be little or no *empirical* concerns regarding *benzoic acids* (or, in general, *arylcarboxylic acids*). However, the mentioned high rate of glycation by 4-bromobenzoic acid-AG might pose a warning (p. 3).

Finally, apart from pure RM issues arising from CAs as drugs (considering AG and CoA formations as normal RMs), other types of adverse drug reaction mechanisms are known or suspected to be caused by compounds that are both lipophilic and anionic (in particular causing liver cholestasis): these are inhibition of transporters/membrane pumps such as BSEP (bile salt export pump) and MRP2 (multidrug resistance-associated protein 2). The many kinds of symptoms of liver injury caused by NSAIDs might suggest that [multifactorial mechanisms](#) provide the rule, not the exception.



Based on the many indications of adverse effects from lipophilic carboxylic acids we have tentatively introduced into SpotRM a new structural alert “Lipophilic carboxylic acid” with fasiglifam as the prime example. We welcome reactions from our users.

(March 2022)