A robust, mechanistically-based *in silico* structural profiler for hepatic cholestasis

James W. Firman[1], Cynthia B. Pestana[1], James F. Rathman[2,3], Mathieu Vinken[4], Chihae Yang[2], Mark T.D. Cronin[1,*]

[1]School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF, UK

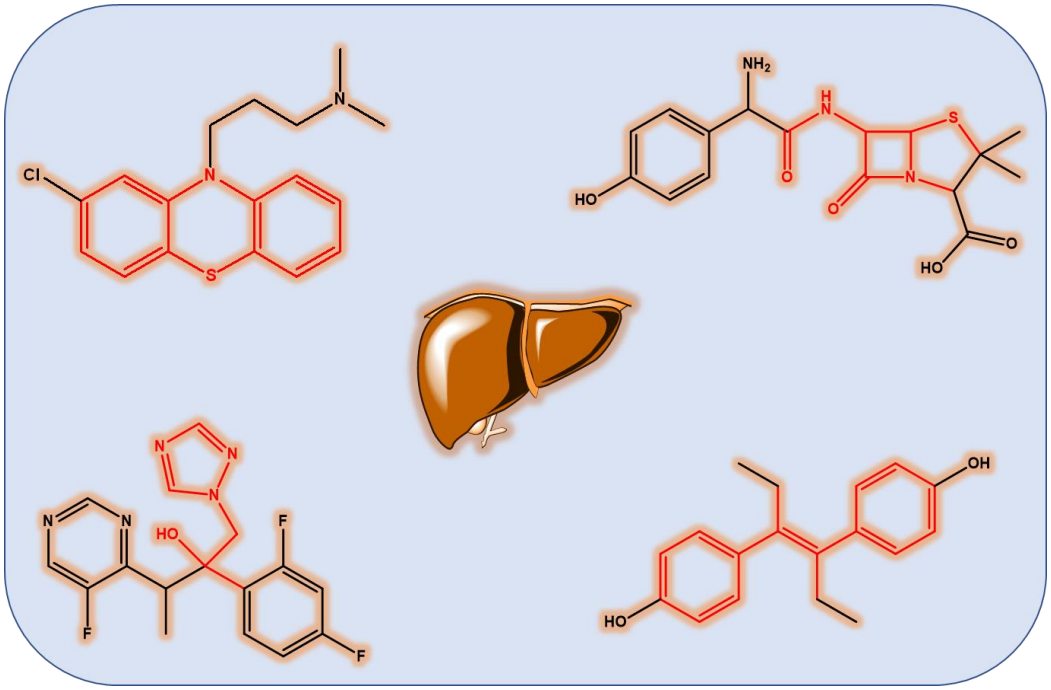
[2]MN-AM, Neumeyerstraße 28, 90411 Nuremberg, Germany; Columbus OH 43235, USA

[3]Chemical & Biomolecular Engineering, The Ohio State University, Columbus OH 43210, USA

[4]Department of *In Vitro* Toxicology and Dermato-Cosmetology, Vrije Universiteit Brussel, Brussels, Belgium

*Author for correspondence:

Mark Cronin, School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF, United Kingdom. E-mail: m.t.cronin@ljmu.ac.uk



Graphic for table of contents use

Abstract

Owing to the primary role which it holds within metabolism of xenobiotics, the liver stands at heightened risk of exposure to, and injury from, potentially hazardous substances. A principal manifestation of liver dysfunction is cholestasis – the impairment of physiological bile circulation from its point of origin within the organ to site of action at the small intestine. The capacity for early identification of compounds liable to exert cholestatic effect is of particular utility within the field of pharmaceutical development, where contribution towards candidate attrition is great. Shortcomings associated with present *in vitro* methodologies forecasting cholestasis render their predictivity questionable, permitting scope for adoption of computational toxicology techniques. As such, the intention of this study has been to construct an *in silico* profiler, founded upon clinical data, highlighting structural motifs most reliably associated with the endpoint. Drawing upon a list of greater than 1500 small molecular drugs, compiled and annotated by Kotsampasakou and Ecker, we have formulated a series of fifteen structural alerts. These describe fragments intrinsic within distinct pharmaceutical classes including psychoactive tricyclics, beta-lactam antimicrobials and oestrogenic/androgenic steroids. Description of the coverage and selectivity of each is provided, alongside consideration of underlying reactive mechanisms and relevant structure-activity concerns. Provision of mechanistic anchoring ensures that potential exists for framing within the adverse outcome pathway (AOP) paradigm – the chemistry conveyed through the alert in particular enabling rationalisation at the level of the molecular initiating event (MIE).

Keywords: cholestasis; structural alert; in silico; toxicity; prediction

1. Introduction

Owing to its primary role within the metabolism of xenobiotics, exposure of the liver to potentially harmful substances is increased relative to other organs. Drug-induced liver injury (DILI) accordingly functions as a leading contributor to developmental attrition and market withdrawal amongst pharmaceuticals.[1] Early identification of compounds liable to induce such adverse effects would be of clear benefit, and as such the development of predictive methods – both *in vitro* and *in silico* – has emerged as a focus of great interest.[2]

It is acknowledged that DILI may manifest itself in one of two primary forms: hepatocellular or cholestatic (in addition to a mixed variety, incorporating characteristics of both).[3] Cholestatic injury specifically arises from impairment to the normal circulation of bile from its site of genesis in the liver to its point of action within the small intestine. Whilst varieties of the disease may emerge as a consequence of physical impediment to bile motion – termed "obstructive cholestasis" – it is the form arising from chemical interference with the physiological systems underlying formation and passage of bile which is of relevance when considering cholestasis stimulated specifically by drugs. Clinical manifestation of drug-induced cholestasis can typically be further classified into one of two archetypal forms: the "bland" (or "pure") variant, emerging from direct impairment of the functioning of hepatic transport proteins responsible for motion of bile, or the mixed presentation, incorporating hepatitis, which is generally idiosyncratic in nature and held to be associated with hypersensitivity reaction.[4] Acute or chronic forms of either may occur, with the latter linked to complications including bile duct destruction and cholangitis.[5]

Induction of bland cholestasis arises as a consequence of interference with the activity of the aforementioned hepatically-expressed transporter systems.[6] As outlined within Figure 1, post-circulation uptake of bile components (including unconjugated bile acids, bilirubin etc) from portal blood into the hepatocyte occurs owing to the action of several basolaterally-located transport proteins – most notably the sodium-taurocholate co-transporting polypeptide (NTCP) and members

of the organic anion transporting polypeptide (OATP) class.[7] Secretion of bile salts, conjugated bilirubin and minor fatty acid substituents into the bile canaliculi is mediated by a still greater number of such transporters, of which the most prominent are those belonging to the of the ATP-binding cassette (ABC) family. Amongst this number are the bile salt export pump (BSEP) and multi-drug resistance-associated protein 2 (MRP2).[8] A complex regulatory system, incorporating (amongst other influences) the oestrogen (ER) and farnesoid X receptors (FXR), mediates functioning of this network.[9, 10] Oestrogenic and androgenic steroids, alongside their derivatives, are most commonly linked with the emergence of this form of disease.

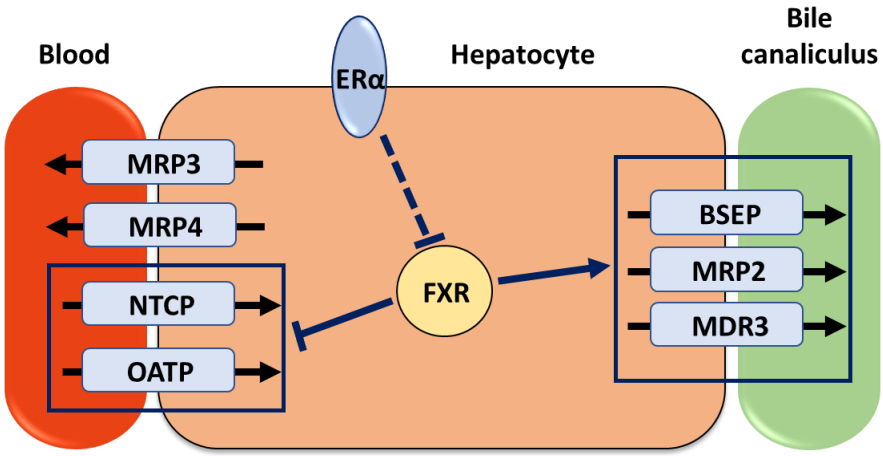


Figure 1. Overview of systems integral within intrahepatic transport of bile components. Uptake from portal blood is mediated by OATP and NTCP transporters, whilst extrusion into bile canaliculi occurs through the action of proteins of the ABC family (most prominently BSEP). Both processes are under regulation of the FXR – the former negatively, the latter positively – with the net impact of maintaining hepatocellular bile concentration at physiological level. Hypothesised repression of FXR owing to oestrogen receptor cross-talk is depicted, as is the presence of two further transporters (MRP3 and MRP4) held to assist in transferral of bile substituents to the bloodstream.

By contrast, the mechanisms underpinning emergence of mixed cholestatic-hepatitis are known less definitively. In numerous instances, evidence points to the relevance of idiosyncratic toxicity – typically associated with genetic variants controlling metabolite generation or immune response.[11, 12] A generalised pathway for idiosyncratic immuno-allergic reaction, as depicted within Figure 2, would centre first upon the formation by hepatic enzymes (commonly of the cytochrome P450 family) of reactive drug metabolites. Inability to adequately detoxify these species permits their adduction of

cellular macromolecules, at which point they may function as haptens.[13] Presentation of such altered peptides either by wild-type or by variant major histocompatibility complex (MHC) isoforms stimulates the recruitment of T cells, thereby inducing inflammation or alternatively cell death. Since this is a sequence of events typically dependent upon the possession of rare enzymatic or MHC genotypes, its occurrence is sporadic (commonly arising in fewer than 1% of patients).[14] Furthermore, it is unpredictable based upon the pharmacology of the substance, with emergence unrelated to dose. Allergic symptoms – including rash and eosinophilia - may be present. An assortment of drug classes, varying substantially with respect both to their structural characteristics and their mode of pharmacological action, are linked with this manifestation of disease. Amongst these are tricyclic psychoactives, macrolide antibiotics, azole antifungals and derivatives of penicillin.[3] Chronic disorder can further incorporate ductopenia as a precursor to vanishing bile duct syndrome – a notable progression in severity.

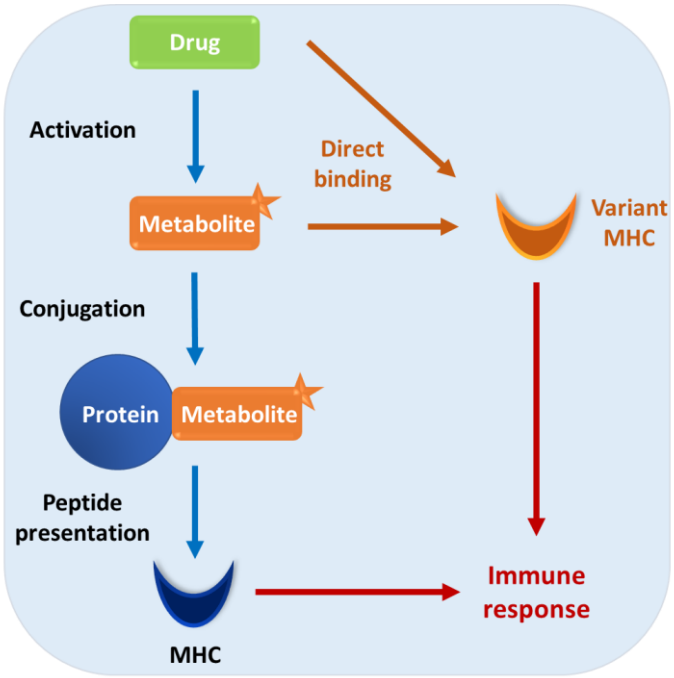


Figure 2. Depiction of proposed mechanisms through which hepatic drug hypersensitivity response may be initiated. Metabolic activation (generally enzymatic) of parent compound to yield reactive species functions as an essential first step. These may proceed to form protein conjugates, which in turn hold capacity trigger immune response following presentation on MHC complexes. Alternatively, either metabolites or parent compound may bind directly at variant MHC or T-cell receptor isoforms, in turn influencing T-cell recruitment and immune activity ("p-I" hypothesis).

Efforts directed at constructing techniques enabling the flagging of compounds liable to stimulate cholestasis have focused primarily upon employment of *in vitro* bioassays examining inhibitory potential at hepatic bile-component transporters.[15-17] However, the extent of the translatability of these findings to the clinical setting remains subject to speculation.[18] Furthermore, limitations exist with respect to the forecasting of toxicity in classes associated with induction of hypersensitivity reaction. Whilst research continues in driving forward understanding of the cellular mechanisms through which drug-induced cholestasis may arise, the complexity of the endpoint and of the pathways contributing to it ensures that this remains a challenging endeavour both conceptually and practically.[19]

In silico, or computational, modelling presents a range of options as regards the addressing of liver toxicity – including either the direct prediction of adverse effect, prioritisation of compounds for further testing or alternatively as contribution towards a scientific "weight of evidence" judgment. The techniques that may be applied range from the use of read-across, through quantitative structure-activity relationships (QSARs) and machine learning.[2] Various factors contribute to ensuring that the structure-based prediction of cholestasis remains a complex endeavour – not least the lack of standardised assays and paucity of accessible robust databases relating to the endpoint, compounded by the current incomplete understanding of mechanisms of action underlying it.[20] As such, relatively few *in silico* models focused solely upon cholestasis have been created.[21-23] A notably powerful tool within computational toxicology is the use of chemical structural alerts – a particular advantage being that alerts can often be understood directly in the context of the molecular initiating event (MIE).[24, 25] Not only may the MIE be employed in informing and validating the alert, but additional scope exists for drawing of support from new approach methodology (NAM) data. Whilst there has accordingly been a rich history of their use in various aspects relating to liver toxicity (both general and specific), a robust series of alerts for cholestasis has yet to emerge.[26-29]

This study aimed to construct such a series of structural alerts describing occurrence of drug-induced cholestasis. For this purpose, the dataset compiled by Kotsampasakou and Ecker, consisting of more than 1,500 small molecular drugs labelled definitively for their association with cholestatic liver injury within a clinical setting, was considered.[21] Through it, we were able to construct a sum of fifteen alerts, covering a variety of therapeutic classes commonly linked to the endpoint. Their selectivity is discussed, alongside the relevance of discernible structure-activity relationships.

2. Materials and methods

2.1. Curation of data

Data employed in construction of alerts were drawn from the listing compiled by Kotsampasakou and Ecker.[21] A total of 1,904 substances were present initially, annotated with binary judgment describing their clinical cholestatic potential. Following manual removal of polymers, mixtures, inorganic salts, organometallic complexes and all compounds having a molecular weight in excess of 1,500, a reduced set consisting of 1,571 distinct small molecules remained (337 positive for cholestasis, 1,234 negative). Existing SMILES strings were edited in order to remove indicators of stereochemistry, before they were canonicalised within the KNIME software (version 4.1; KNIME, Zurich, Switzerland) through use of the "RDKIt Canon SMILES" node (RDKit; www.rdkit.org). Substances were subsequently mapped to COSMOS ID (CMS ID) and CAS RN. Complete listings, annotated in accordance with alert matches, are available within Supplementary Table 1.

2.2. Development of structural alerts

The chemical structures of each of the 1571 compounds within this final dataset were examined. Presence of common, shared molecular fragments discriminating cholestasis-positive entries from cholestasis negative was sought. In order to achieve this, manual judgment was employed.

Mechanistic rationale behind alerts was sought through means of an extensive literature search, with structure-activity relationship forming a particular focus of attention.

2.3. ToxPrint chemotype analysis

ToxPrint chemotypes were generated using the publicly-available ChemoTyper application (version 1.0; Molecular Networks, Erlangen, Germany) in order to detect building blocks which may be suitable in forming the basis of structural alerts. Selected chemotypes were drawn from those initially reported within Yang et al., and further expanded by Rathman et al.[30, 31] Selectivity with respect to occurrence in cholestasis-positive compounds was quantified through determination of Z-score, as derived in accordance with protocols described previously.[24, 31] Identical analysis was performed upon a selection of 305 marketed pharmaceuticals positive for generalised DILI, sourced from Rathman et al. and referred to henceforth as the "human DILI" set. [31]

2.4. Quantification of structural alert performance and selectivity

For each alert, quantification of selectivity was achieved through calculation of both of odds ratio (OR) and positive predictivity value (PPV).[32] Employing inputs outlined within Table 1, each was derived in accordance with the formulae depicted respectively In Equations 1 and 2.

		Predicted	
	Outcome	Cholestatic	Non-cholestatic
Experimental	Cholestatic	TP	FN
	Non-cholestatic	FP	TN

Table 1. Contingency table describing identities of inputs employed within quantification of selectivity, where TP represents number of true positives, FN the number of false negatives, FP number of false positives and TN number of true negatives.

$$Odds\ ratio = \frac{TP * TN}{FP * FN} \qquad eq\ (1)$$

Equation 1: Formula for calculation of odds ratio (identity of variables as described within Table 1).

$$Positive\ predictvity\ value = \frac{\boldsymbol{TP}}{\boldsymbol{FP} + \boldsymbol{TP}} \qquad eq\ (2)$$

Equation 2: Formula for calculation of positive predictivity value (identity of variables as described within Table 1).

3. Results and discussion

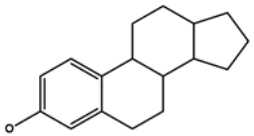
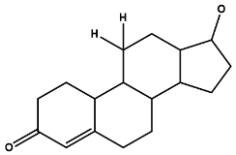
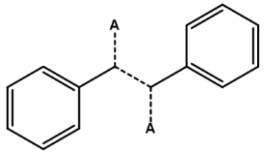
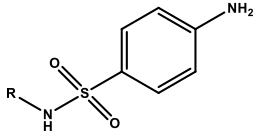
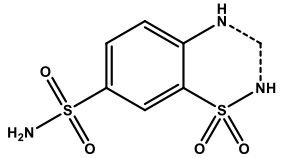
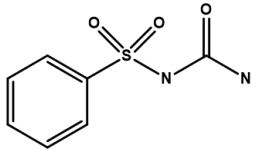
3.1. Overview of compiled structural alerts

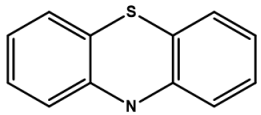
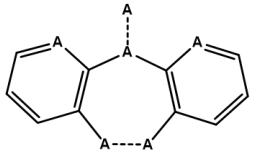
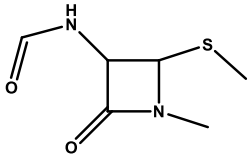
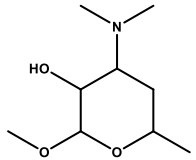
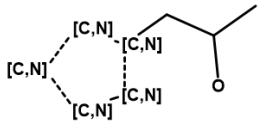
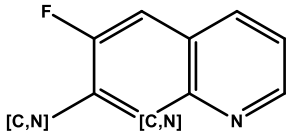
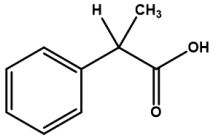
Fifteen structural alerts related to onset of clinical cholestasis were constructed and are depicted in Table 2. Accompanying them are data concerning coverage and apparent selectivity – the latter expressed in the form of odds ratios and positive predictivity values, as determined through methods described within Section 2.3. These were further grouped loosely in accordance either with common pharmacological profile or with shared chemical features (steroid receptor modulator, sulfonamide etc.). Extent of coverage varied, with the broadest alert (antibiotic beta-lactam core) capturing a total of 50 compounds and the most narrow (stilbene derivatives) six. Degree of selectivity was similarly diverse: the desosamine moiety was represented within twelve cholestasis-positive compounds and only a single cholestasis-negative, whereas by contrast the fluoroquinolone core appeared within three positives and thirteen negatives.

Where possible, rationalisation behind the apparent non-manifestation of cholestasis within compounds bearing the key structural features (thereby reducing selectivity) is offered. It should be noted, however, that a variety of factors distinct from simple possession of the fragment may influence the apparent occurrence of adverse outcome in the clinical setting. Broadly, these may be considered determinants of drug exposure: it of course stands to reason that, should a compound have been subject to limited use in patients, then the potential for emergence of identifiable cholestasis shall remain reduced. Amongst the fluoroquinolone family are a number of agents which, on account of toxicity, either underwent rapid market withdrawal or otherwise saw their use severely restricted.[33] Alongside these are pharmaceuticals of various forms which grew to become underused owing to development of counterparts displaying comparatively favourable efficacy, tolerability and pharmacokinetic profiles. Numerous drugs have, furthermore, found exclusive niches within veterinary medicine – including several antimicrobial sulfonamides and some phenothiazines.[34, 35] Alternatively, compounds within common use shall exhibit reduced likelihood of inducing DILI should

their route of administration be other than oral. Examples of such are present amongst the sulfonamides (sulfacetamide and sulfabenzamide) and antihistamines (olopatadine) formulated solely for topical use.[36, 37]

Discussion related to the mechanistic and structure-activity aspects present within each category is provided within Section 3.3 (refer to column titled “Postulated MIE” within Table 2 for overview). Data concerning the identity of compounds matching each alert are reported in Supplementary Table 1.

Alert title	Defining structure	Postulated MIE	Compound matches		Selectivity score	
			Chol. positive	Chol. negative	OR	PPV
Steroid receptor modulator						
Oestrogenic steroid		Binding at ER	8	4	7.48	0.67
Androgenic steroid		Undefined	4	6	2.46	0.40
Stilbene derivative		Binding at ER, protein alkylation, haptenation (post-activation)	4	2	7.40	0.67
Sulfonamide						
Sulfonamide (antimicrobial)		Protein nitrosylation, haptenation (post-activation)	4	18	0.81	0.18
Thiazide		Protein nitrosylation, haptenation (post-activation)	17	2	32.73	0.89
Benzene-sulfonylurea		Protein acylation, haptenation (post-activation)	9	2	16.90	0.82
Psychoactive tricyclic						

Phenothiazine		Protein alkylation, haptenation (post-activation)	16	7	8.74	0.70
Dibenzo-cycloheptane		Protein alkylation, Haptenation (post-activation)	19	6	12.23	0.76
		Anti-infective				
Beta-lactam		Protein acylation, haptenation	29	21	5.44	0.58
Desosamine		Protein nitrosylation, haptenation (post-activation)	12	1	45.53	0.92
Azole antifungal		Undefined	4	5	2.95	0.44
Fluoroquinolone		Direct binding at MHC or T-cell receptor	3	13	0.84	0.19
		Other				
NSAID (-profen)		Protein acylation, haptenation (post-activation)	5	3	6.18	0.63

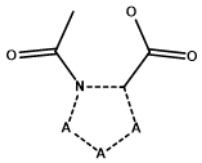
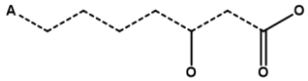
ACE inhibitor (peptidic)		Protein acylation, haptenation (post-activation), antagonism at ACE	7	3	8.70	0.70
Statin		Undefined	7	5	5.21	0.58

Table 2. Key structural features relating to each alert, accompanied by associated postulated molecular initiating event (MIE) and representation amongst cholestasis-positive and cholestasis-negative compounds. Selectivity scores are provided in the form of the odds ratio (OR) and positive predictivity value (PPV).

3.2. Analysis of fragment selectivity through use of ToxPrint chemotypes

Preliminary profiling of the dataset using ToxPrint chemotypes revealed the identities of chemical fragments displaying greatest frequency within (and selectivity for) cholestasis-positive compounds. Listed within Table 3 are the chemotypes most reliably represented amongst this set, ordered in accordance with their computed Z-scores (higher values indicating greater selectivity). Many of these structural features are further present within the manually-developed alerts. Examples include *ring:hetero_[6]_Z_1_2_4-* (a feature of thiazide diuretics), *ring:hetero_[6]_N_triazine_generic* (azole unit present within antifungal class), *ring:hetero_[6_6_6]_N_S_phenothiazine*, (phenothiazine heterocycle), *bond:S(=O)N_sulfonylamide, bond:S(=O)N_sulfonamide* (generic sulfonamide) and *ring:hetero_[4]_N_beta_lactam*, *ring:hetero_[4]_N_azetidine* (beta-lactam antibiotics). An unabridged listing of the outcomes of this analysis may be found within Supplementary Table 2.

Chemotype name	Distribution	Z-score
ring:hetero_[6]_Z_1_2_4-	0.79	7.20
bond:N[!C]_amino	0.60	6.38
ring:hetero_[6]_N_triazine_generic	0.67	6.35
bond:CS_sulfide_dialkyl	0.41	5.81
bond:CS_sulfide	0.37	5.61
ring:hetero_[6_6_6]_N_S_phenothiazine	0.68	5.60
group:ligand_path_5_bidentate_propandiamine	0.50	4.82
bond:CN_amine_aromatic_generic	0.31	4.81
ring:hetero_[4]_N_beta_lactam	0.46	4.80
ring:hetero_[4]_Z_generic	0.45	4.70
ring:hetero_[4]_N_azetidine	0.45	4.69
bond:S~N_generic	0.38	4.66
bond:S(=O)N_sulfonylamide	0.38	4.62
bond:S(=O)N_sulfonamide	0.38	4.48
ring:hetero_[5]_N_O_isoxazole	0.61	4.31

Table 3. ToxPrint chemotypes present with greatest selectivity within cholestasis-positive compounds (ranked according to Z-score). Distribution metric describes the proportion of compounds containing a given alert that are also positive for cholestasis

Chemotype distribution amongst the cholestasis dataset was subsequently compared against that within a further selection of pharmaceuticals judged for their capacity to induce generalised hepatotoxicity (the “human DILI” set). Outcomes are depicted within Figure 3. It is apparent that, as

anticipated, many of the fragments definitively associated with cholestasis are likewise overrepresented amongst the DILI-positive compounds. Conversely, there are several registering notably higher Z-scores for general liver toxicity. It can be hypothesised that these, amongst which appear *hetero_[6]_pyrimidine*, *hetero_[5]_furan* and *carboxamide_generic*, feature prominently within molecules exerting adverse effects through non-cholestatic routes.

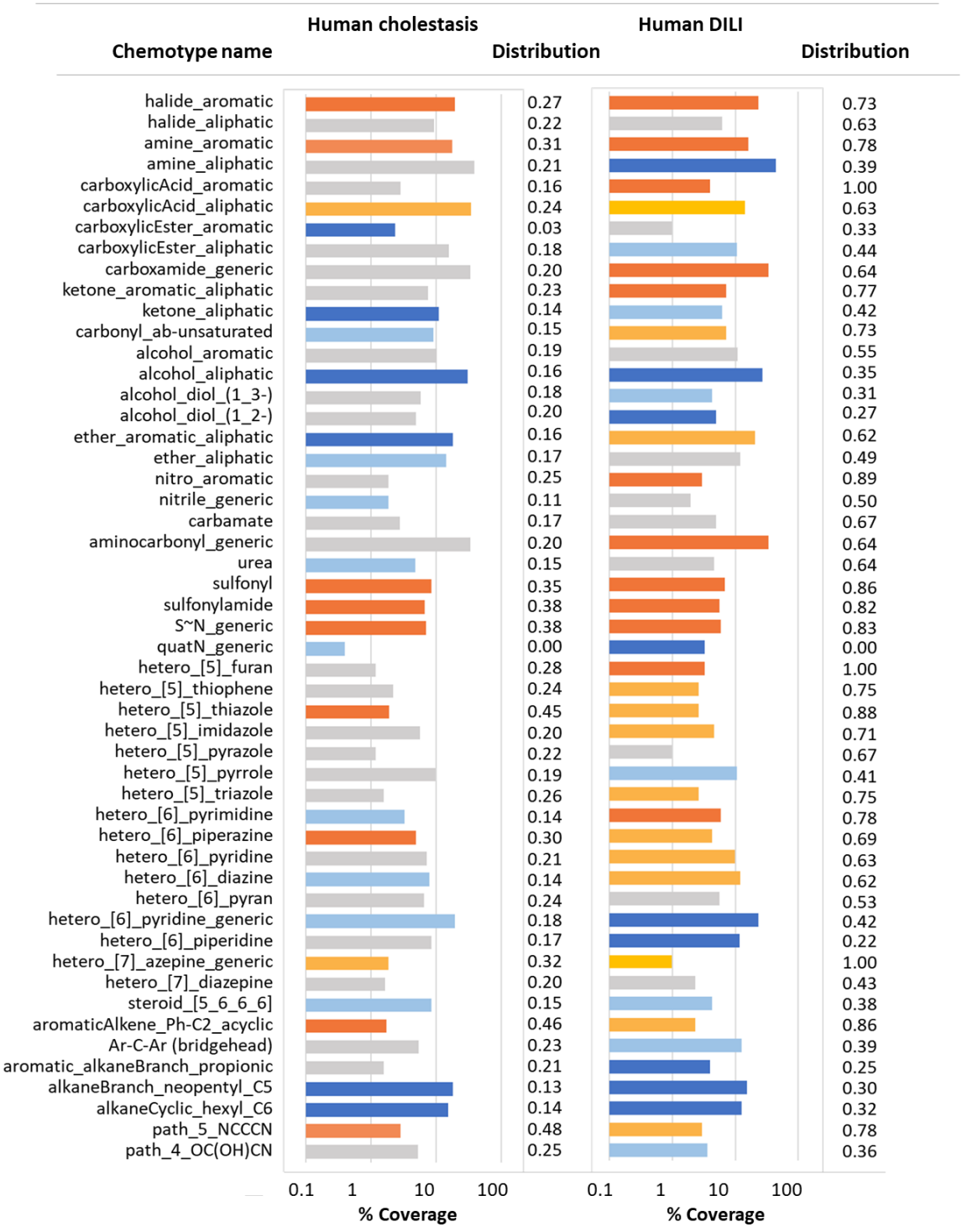


Figure 3. ToxPrint chemotype analysis of cholestasis and general DILI datasets. Bars are colour-coded in accordance with Z-scores: dark orange ($Z \geq 2$), orange ($1 \leq Z < 2$), grey ($-1 < Z < 1$), light blue ($-1 \leq Z < -2$), and dark blue ($Z \leq -2$). Length represents frequency of matches (% of structures in dataset containing given chemotype), whilst the Distribution metric describes the proportion of compounds containing a given alert that are also positive for cholestasis

3.3. Alert descriptions

3.3.1. Steroid receptor modulator

3.3.1.1. Oestrogenic steroid

Oestrogenic steroids and their derivatives are employed within birth control formulations, in hormone replacement therapy and in treatment of specific cancers. Through construction of an alert capturing the characteristic phenolic A-ring substituent within a tetracyclic steroid core, twelve such compounds from within the dataset were recovered. Eight were acknowledged as being causative of cholestasis – including oestradiol and its esters, alongside ethinylestradiol and estropipate. Evidence exists associating the influence of ER agonism with downregulation of BSEP and related transport proteins, through mechanisms reliant upon cross-talk with farnesoid X receptor (FXR) signalling and repression of gene expression (relevant similarly within intrahepatic cholestasis of pregnancy).[38, 39] Given the form of bland, non-inflammatory cholestasis induced through administration of these compounds, it is highly probable that such a pathway holds at least partial responsibility. Extent of oestrogenicity may, therefore, play a decisive role in determining the extent to which emergence of cholestasis through this route is likely to occur. Each of the eight "positives", as oestradiol prodrugs or analogues, may be anticipated to exert particularly strong oestrogenic effect.

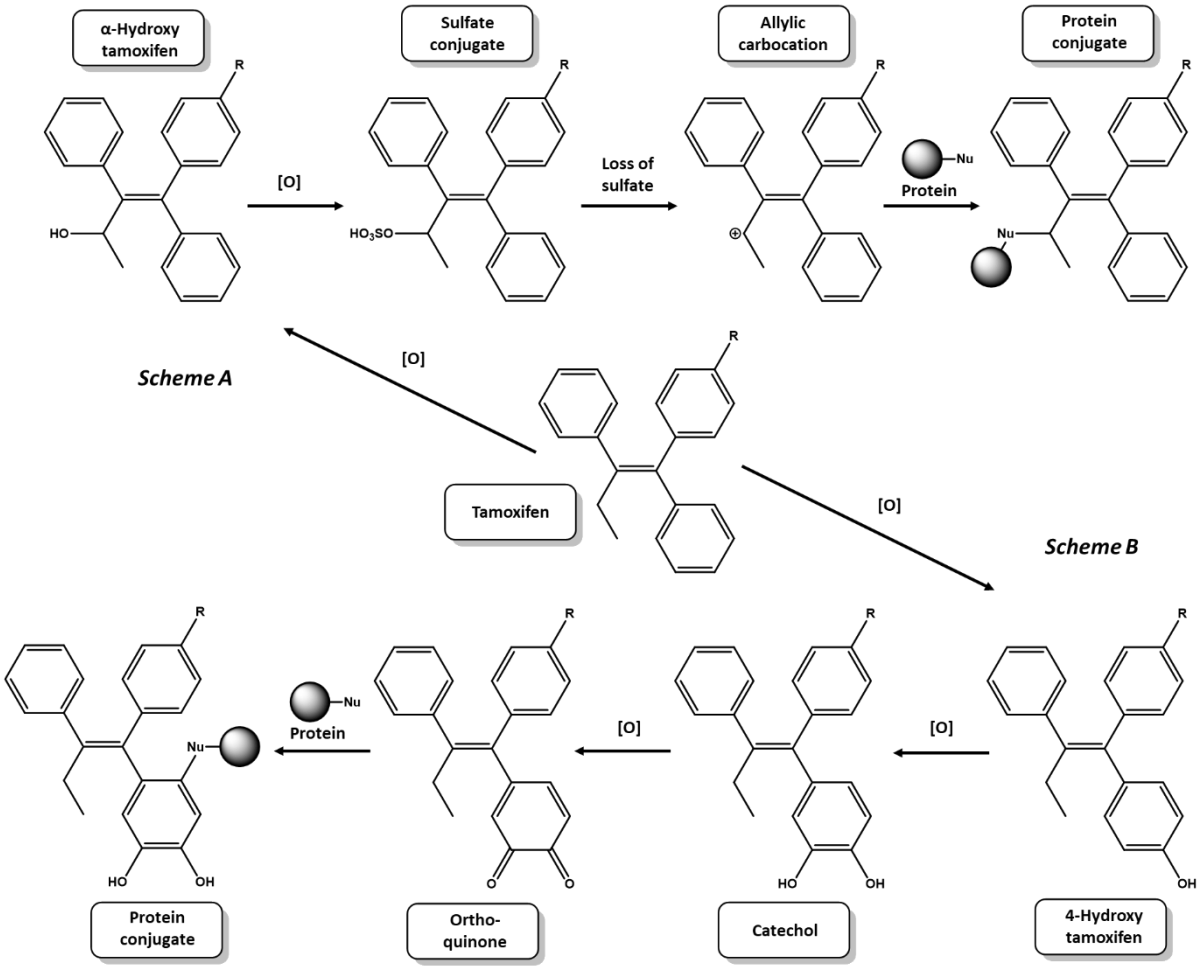
3.3.1.2. Androgenic steroid

In a similar manner to their aforementioned oestrogenic counterparts, androgenic steroids deployed for medicinal purpose have been observed to induce a form of bland cholestasis within susceptible patients.[40] The pathway underlying is largely undefined, although course of presentation would suggest that perturbation of aforementioned proteins integral within the transport of bile and its

constituents lies central. Studies have highlighted tentative associations between aberrant expression of such proteins, and heightened vulnerability towards androgen-stimulated cholestasis – these being specifically mutations within the genes encoding for BSEP and ATP8B1, and haploinsufficiency in the pregnane X receptor (PXR).[41, 42] Ten compounds triggered the relevant alert – of which four were positively judged causative of cholestatic injury.

3.3.1.3. Stilbenoid

Numerous derivatives of stilbene are noted for holding xenoestrogenic capacity.[43] Accordingly, this motif has been adopted as the basis for a class of selective oestrogen receptor modulators (SERMs), typified by tamoxifen and toremifene, which find use in treatment of hormone-responsive cancers and infertility. Although steatosis is the more common manifestation of hepatotoxicity arising through these compounds, cholestasis has been observed within four of the six members recovered from the dataset. Both bland and inflammatory varieties of the disease have been noted to occur – each likely having its own distinct mechanistic origin. The former can reasonably be explained in terms of intrinsic activity at the ER, with evidence suggesting that, in a manner analogous to that of steroidal oestrogens, tamoxifen in particular is capable of inducing a marked downregulation in BSEP expression *in vitro*.[44] By contrast, inflammatory cholestasis is generally idiosyncratic in nature, originating in aberrant reactive metabolite-triggered immune response (i.e. hypersensitivity) secondary to hepatic protein adduction. The propensity for tamoxifen to generate such contributing species – in the form of an allylic carbocation and ortho-quinone intermediates – is acknowledged.[45] Portrayed within Figure 4 are hypothesised routes towards the creation of these: carbocations arising as a consequence of cytochrome P450-catalysed α-hydroxylation and subsequent sulfation (Scheme A), and ortho-quinones through enzymatic hydroxylation yielding catechol (Scheme B).[46, 47]



297

298 **Figure 4.** Competing routes towards generation of hypothesised reactive tamoxifen derivatives, each with
299 apparent potential to trigger hepatoxicity secondary to adduction of proteins at nucleophilic amino acid
300 residues. Scheme A depicts formation of allylic carbocation following α-hydroxylation and subsequent loss of
301 sulfate. Outlined within Scheme B is creation of catechol arising through two-stage hydroxylation of aromatic
302 unit, which may in turn undergo oxidation to yield ortho-quinone.

303

304 ## 3.3.2. Sulfonamide

305 ### 3.3.2.1. Sulfonamide (antimicrobial)

306 This alert relates to the para-aminobenzene sulfonamide moiety present within "sulfa" agents
307 typically utilised for their bacteriostatic properties. Owing to the symptom profile of the liver injury –
308 which is suggestive of hypersensitivity response – a shared mechanistic origin with that of general
309 sulfonamide allergy may be considered plausible.[48] Research indicates that it is the amine unit
310 situated para- to the sulfonamide group which is pivotal within generation of reactive derivatives liable
311 to constitute antigenic determinants, serving as it does as a locus for oxidation leading to emergence

of an electrophilic nitroso group capable of generating protein adducts (Figure 5).[49] Possession of this feature was found within 22 compounds present in the dataset – only four of which (including sulfamethoxazole and sulfasalazine) held association with cholestasis. It has been hypothesised that co-presence of a five-membered or six-membered aromatic heterocycle bound at the sulfonamide nitrogen may contribute to the immunogenicity of the protein conjugate. However, such a unit is common amongst the 18 apparently non-cholestatic compounds – and is furthermore absent in "positives" carbutamide and furosemide. As such, delineation of secondary structural characteristics associated definitively with outcome is not at present possible. It should be added however that, as alluded to within Section 3.1, usage practices may account for many of the overt "negatives".

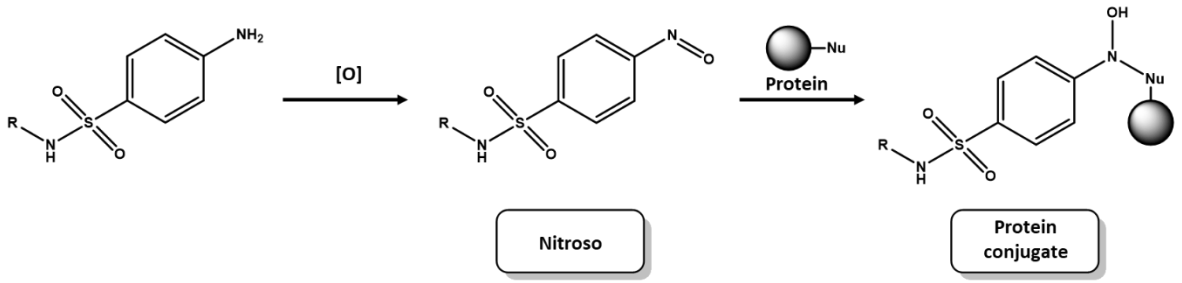


Figure 5. Oxidation of amino moiety positioned para- to sulfonamide unit, producing reactive nitroso metabolite.

3.3.2.2. Thiazide

Functionalisation of benzothiadiazine produces a series of sulfonamides possessing diuretic effect. Their structure is highly distinctive, holding as it does two sulfonamide units: one exposed, the other cyclic. Occurrence of cholestasis has been recorded within seventeen of the nineteen compounds bearing this core, indicating a high degree of consistency with respect to association. Symptoms of hypersensitivity are typically present, suggesting an idiosyncratic origin to disease onset.[50] The molecular mechanisms underlying this response remain undefined, with tendency towards metabolism apparently varying across the class .[51] Recovery of 2,4-disulfamyl-5-trifluoromethylaniline (DTA) following hydroflumethiazide dosing suggests that opening of the thiadiazine ring is possible, exposing in the process a primary amine positioned para- to the free sulfonamide moiety (Figure 6, Scheme A).[52] Such a group could function analogously to that present

within the aforementioned antimicrobial sulfa compounds, serving as a focal point for oxidation and subsequent formation of the reactive nitroso. Alternatively, the generation of a distinct nitrosamine derivative of hydrochlorothiazide has been demonstrated *in chemico* – although the relevance of this to the *in vivo* setting remains uncertain (Figure 6, Scheme B).[53] Lack of clarity concerning the route towards activation ensures the apparent non-occurrence of cholestasis within polythiazide and methyclothiazide may not readily be rationalised. It should be noted, however, that unlike the seventeen cholestasis-positive compounds, these each exhibit methylation at the benzothiadiazine 2-position – the impact of which with respect to the metabolism or to the pharmacokinetic properties of this class has yet to be determined.

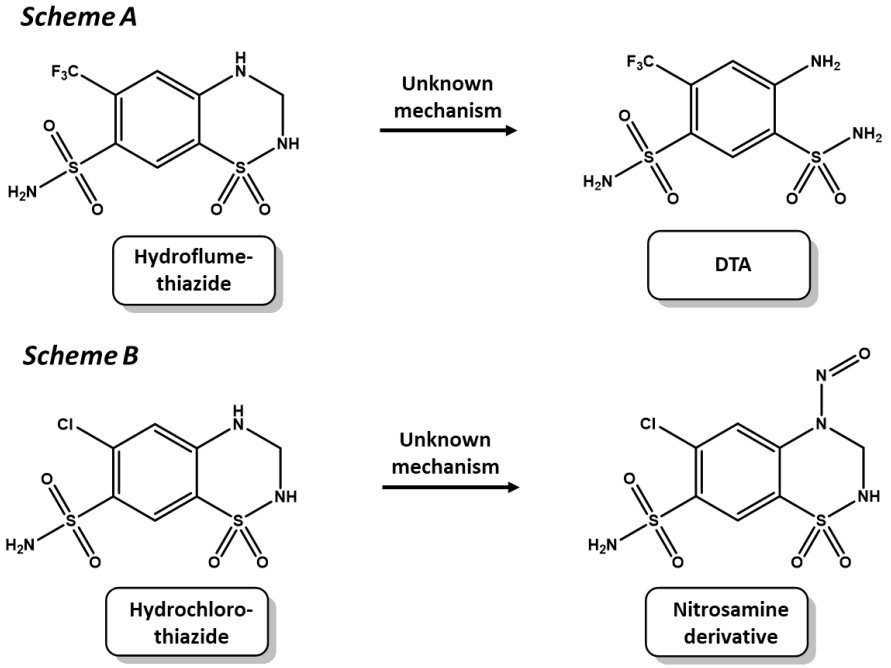


Figure 6. Occurring through an uncharacterised mechanism, transformation of hydroflumethiazide yielding 2,4-disulfamyl-5-trifluoromethylaniline (DTA) – a para-amino sulfonamide – has been reported in man (Scheme A). Depicted within Scheme B is the generation, *in chemico*, of a hydrochlorothiazide nitrosamine derivative.

3.3.2.3. Benzenesulfonylurea

A further sulfonamide derivative, this unit characterises a class of antagonists at the pancreatic ATP-sensitive potassium channel widely employed within treatment of diabetes. Nine of the eleven compounds holding this structural core – including tolbutamide, chlorpropamide and glibenclamide – were judged positive for association with cholestasis. Symptoms suggestive of idiosyncratic reaction

are typically present.[50] Tentative evidence has emerged implying that cleavage about the urea unit (occurring through a currently undefined mechanism) might precede formation of isocyanate species vulnerable towards nucleophilic attack by thiol-containing peptide residues, in turn leading to the familiar protein adduction (Figure 7).[54] Each of the two recovered class members apparently not causative of cholestatic injury – glisoxepide and gliquidone – bear particular structural similarity to fellow "second generation" sulfonylurea medications glimepiride, glipizide and glibenclamide.

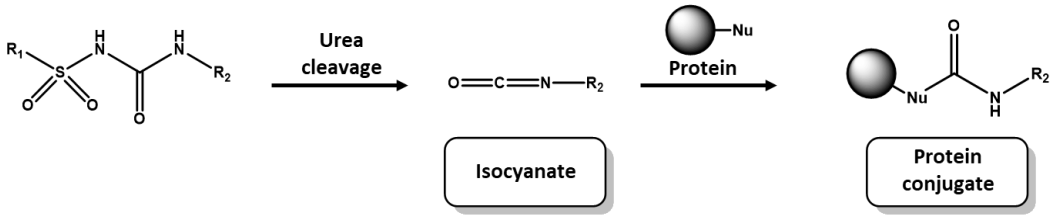


Figure 7. Cleavage of parent molecule about urea, occurring through an undefined mechanism, yielding potentially reactive isocyanate.

3.3.3. Psychoactive tricyclic

3.3.3.1. Phenothiazine

The tricyclic phenothiazine moiety is associated with a range of bioactivities, forming a key constituent of molecules acknowledged as interacting with varying potencies across dopaminergic, serotonergic, adrenergic, cholinergic and histaminergic receptors. Many such compounds have been employed in clinical settings either for their antipsychotic effect, or for the relief of allergy-related symptoms. A total of 23 molecules possessing the alert were retrieved, of which sixteen were positive for cholestasis – apparently of the idiosyncratic profile.[55] Amongst these were the typical antipsychotics chlorpromazine and thioridazine, which have been focus of study concerning their potential for transformation into reactive metabolites.[56] Although direct translational relevance remains undetermined, it has been demonstrated that cytochrome P450-mediated aromatic hydroxylation can facilitate the formation of electrophilic quinone-imine intermediates susceptible to thiol adduction (Figure 8).[57] Whilst each recovered molecule containing the relevant alert possesses the structural

feature (unsubstituted 7-position) necessary for this pathway to be initiated, seven are judged not to be causative of cholestasis.

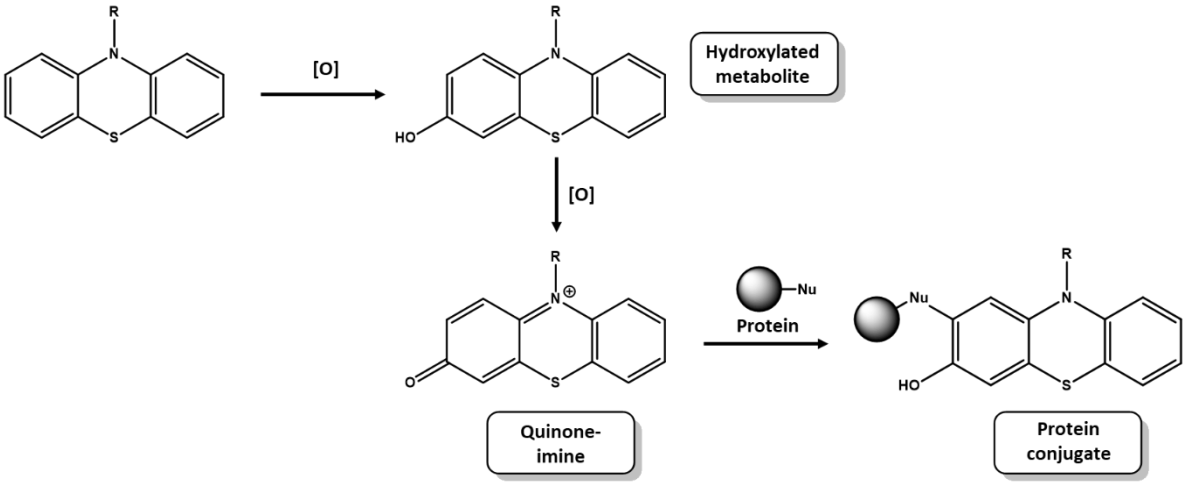


Figure 8. Scheme outlining formation of reactive quinone-imine intermediate following two-step oxidative metabolism of phenothiazine core.

3.3.3.2. Dibenzocycloheptane

Like those amongst the phenothiazine class, dibenzocycloheptanes are capable of modulating activity at neurotransmitter and histaminergic receptors – albeit with an effect profile which tends more towards antidepressant than antipsychotic. A modified equivalent of this motif is further found within the antiretroviral nevirapine. The nature of the alert allows for variation in composition amongst the rings, and in all a total of 25 compounds are found bearing a form of it: nineteen associated with cholestasis (idiosyncratic presentation), six not.[55] In this instance, the enzymatic oxidation of aromatic groups to yield labile epoxides is forwarded as a primary route through which activation of the molecule might occur. Such a biotransformation has been noted to occur both in the antidepressants amitriptyline and nortriptyline, and also in nevirapine (Figure 9, Scheme A).[58-60] An additional locus for epoxidation has been identified in compounds typified by carbamazepine, which incorporate an alkene functionality within the central seven-membered ring (Figure 9, Scheme B).[61, 62] Irrespective of the ultimate site of epoxide formation, apparent capacity to alkylate protein residues remains conserved.

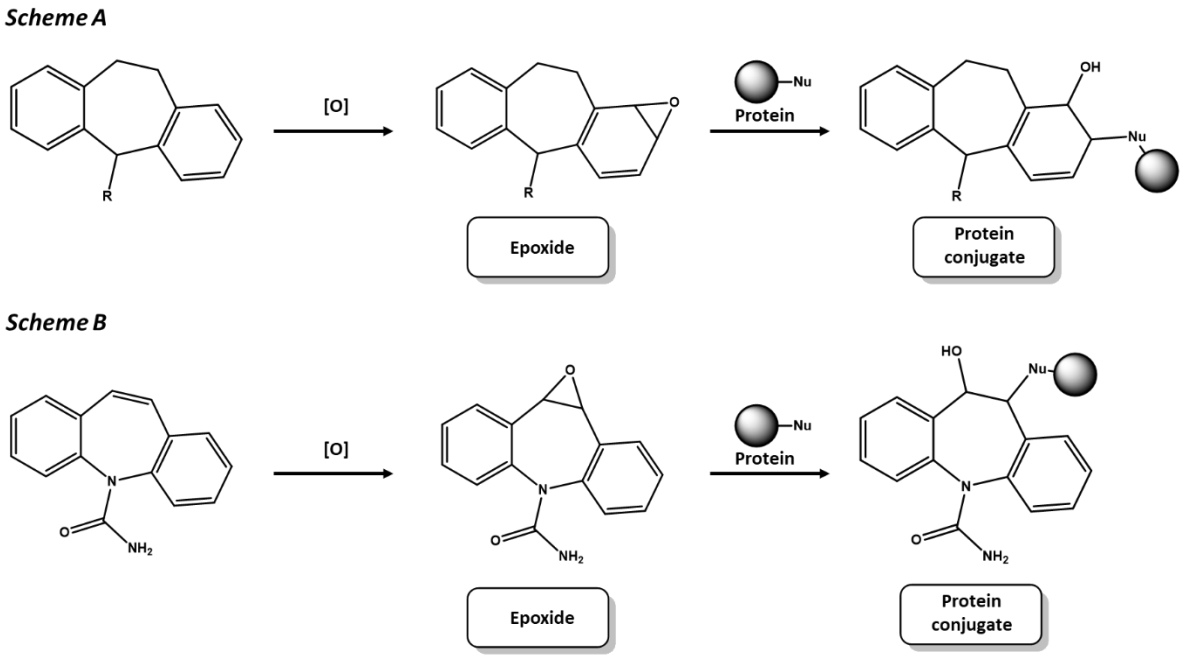


Figure 9. Aromatic epoxidation, as observed within amitriptyline and nortriptyline, yielding reactive epoxide intermediate (Scheme A). Scheme B illustrates competing pathway present in carbamazepine and similar compounds possessing alkene functionality at central ring.

3.3.4. Anti-infective

3.3.4.1. Beta-lactam

The beta-lactam unit forms the pharmacophoric core of an array of structurally-related antibacterials, amongst which are found penicillins and cephalosporins. A total of fifty compounds were noted to bear this motif, constituting the broadest coverage of all present alerts. The emergence of cholestasis is closely associated with hypersensitivity reaction.[63] Indeed, allergic response to such compounds is comparatively common, arising as a consequence of the intrinsic reactivity of the strained azetidinone substructure. Just as the susceptibility of the carbonyl to nucleophilic attack from serine residues upon bacterial transpeptidase accounts for therapeutic utility, so may its ready reaction with hepatocellular proteins precede haptenation and the triggering of inflammation.[64] Such products of ring-opening, known as antigenic determinants, have been characterised.[65] Their capacity to form adducts has been illustrated in penicillin derivatives amoxicillin and flucloxacillin, both of which are actively associated with clinical cholestasis.[66, 67] Displayed within Figure 10, Scheme A is a generalised overview (applicable both to penicillins and cephalosporins) outlining the predominant form which such

conjugates are hypothesised to take. It must be acknowledged, however, that of the fifty molecules identified, 21 were ultimately judged to be without cholestatic association. It has been suggested that the nature of the side-chain, extending beyond the amine functionality, has influence upon the ultimate immunogenic potential of the determinant. Closer examination of the returned compounds enabled identification of substructures more reliably related to onset of liver dysfunction. These include the oxy-imino unit present within cephalosporins such as cefotaxime, and the bio-isosteric phenyl-substituted isoxazole fragment characteristic of penicillin-derivatives amoxicillin and flucloxacillin (Figure 10, Scheme B). Interestingly, this matches very closely the conclusions reached by Hasdenteufel et al., who likewise noted the increased sensitisation potential of pharmaceuticals possessing those features.[68]

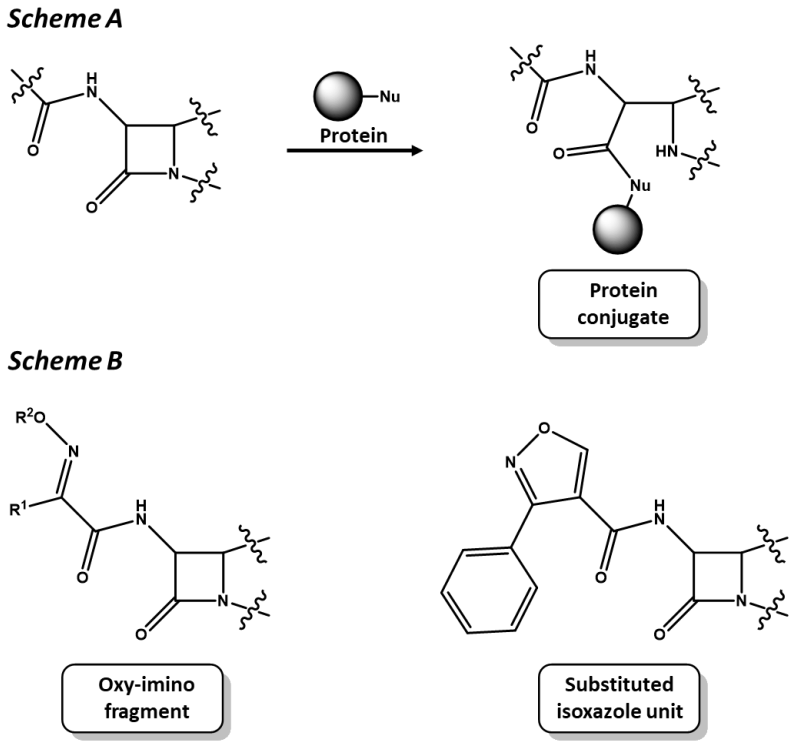


Figure 10. Opening of the intrinsically strained azetidinone ring through nucleophilic addition (Scheme A). Depictions of side-chain features most reliably associated with emergence of cholestasis (Scheme B).

3.3.4.2. Desosamine

Antibiotics of the macrolide class share in common the possession of at least a single desosamine unit, bound through means of an ether linkage to a central macrocyclic lactone core. Of the thirteen

molecules matching the alert (amongst which were erythromycin, clarithromycin and telithromycin), twelve held association with induction of clinical cholestasis. This hepatotoxicity is idiosyncratic in nature, typically manifesting in the form of a mixed cholestatic-hepatitis complete with immuno-allergic features suggestive of hypersensitivity.[48] It has been hypothesised that the origins of this response may lie in two-step, enzymatic biotransformation of the desosamine tertiary amine moiety – forming a reactive nitroso species capable of alkylating susceptible proteins at thiol-incorporating residues (prospective scheme outlined within Figure 11).[63, 69] General similarity of structure amongst these members was observed to be high.

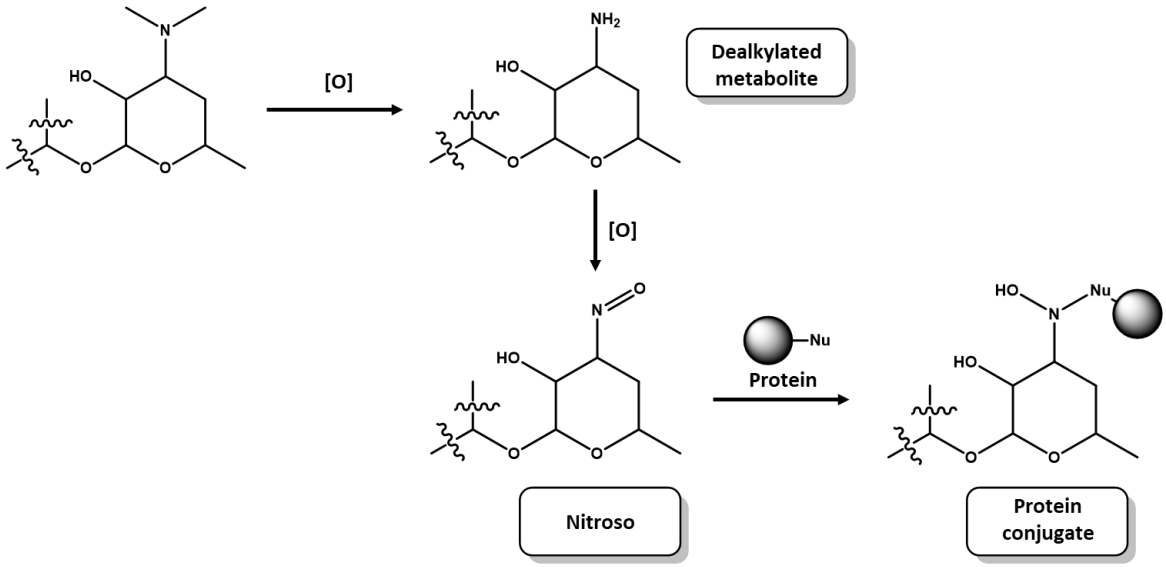


Figure 11. Two-stage oxidative metabolism of desosamine yielding reactive nitroso derivative.

3.3.4.3. Azole antifungal

Representing a class of widely-utilised fungistatic agents characterised by possession of an aromatic, five-membered nitrogenous heterocycle, this alert was matched within nine molecules. Four were positively associated with emergence of cholestasis (idiosyncratic presentation) whereas five were not. It should be noted that each of the former four (itraconazole, posaconazole, fluconazole and voriconazole) are employed generally for treatment of systemic infection, whereas the remaining five (amongst which are terconazole, tioconazole and econazole) are instead prescribed almost exclusively for localised, topical application.[63] It is this distinction in likely hepatic exposure, rather than variation

in intrinsic molecular structural characteristics, which appears to best rationalise variation in proclivity towards induction of cholestasis. Underlying mechanism remains unclear, with apparent variation in tendency towards experience metabolic transformation present between compounds.[70]

3.3.4.4. Fluoroquinolone

The 6-fluoroquinolone unit is the core structural feature of a class of topoisomerase-inhibiting antibiotics. A total of sixteen compounds were recovered bearing this unit, although only four of this number were associated with onset of cholestatic liver injury – those being ciprofloxacin, moxifloxacin, norfloxacin and ofloxacin. On account of the standard symptom profile, it is accepted that origin lies in idiosyncratic hypersensitivity response.[71] Rather than being dependent upon the generation of reactive metabolites, it has instead been hypothesised that the immunogenicity of this grouping arises as a consequence of direct, non-covalent binding of parent compound either to MHC or to T-cell receptors (the "p-I" concept, as outlined within Figure 2).[72-74] Relevant examples of these receptors would ideally have to be characterised before key structural features associated with emergence of adverse response could be definitively placed. As referenced within Section 3.1, the presence of alternative forms of toxicity has ensured that many compounds within this family have been subject only to limited clinical application – likely reducing the scope for onset of recorded cholestasis.

3.3.5. Other

3.3.5.1. NSAID (-profen)

Several commonly-deployed COX-inhibiting anti-inflammatory medications ("profens") incorporate the 2-phenylpropanoic acid backbone which characterises this alert. Eight such compounds were recovered bearing the fragment – five (including ibuprofen, fenoprofen and ketoprofen) linked with clinical cholestasis. For the further three holding no association with the endpoint, mitigating factors relating to usage and exposure can be forwarded: suprofen (for oral administration) and pirprofen saw only limited adoption on account of toxicity-related market withdrawal, whereas carprofen

receives use exclusively in veterinary medicine.[75-77] Disease typically manifests in the form of cholestatic hepatitis, with a frequency and symptom profile strongly indicative of hypersensitivity response.[78] A speculative mechanism through which this might arise has been forwarded, centring upon bioactivation of the carboxylic acid unit to yield potentially reactive acyl glucuronide or coenzyme A conjugates (Figure 12, Schemes A and B respectively). Capacity of these products to adduct hepatocellular proteins has been demonstrated, although the direct relevance of this modification remains undetermined.[79, 80, 81]

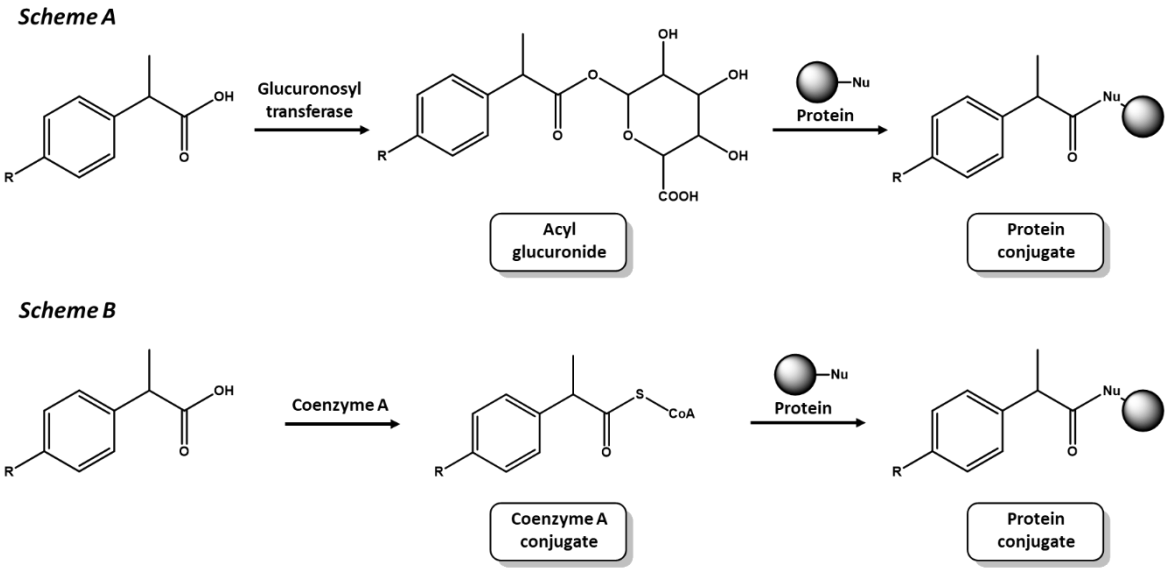


Figure 12. Metabolic transformation of carboxylic acid unit yielding potentially reactive acyl glucuronide (Scheme A) or coenzyme A (Scheme B) functionalities.

3.3.5.2. ACE inhibitor (peptidic)

This alert captures the peptidomimetic core present within vasodilatory angiotensin converting enzyme (ACE)-inhibitors such as captopril, enalapril and lisinopril. Ten compounds were found bearing this motif, amongst which was a single antilipidaemic agent (timofibrate). Seven of this number were linked with clinical manifestation of cholestasis – the presentation of which is apparently highly variable.[50] Little is known definitively concerning the mechanism through which these drugs may invoke liver injury. A number of theories have been posited, some of which may be general to all within class, and others specific to given members. It has been forwarded, for example, that elevated bradykinin levels occurring secondary to ACE inhibition may stimulate increased prostaglandin

synthesis, in turn leading to impairment in bile motility.[82] By contrast, features such as the thiol unit present on captopril, or the proline unit further shared by lisinopril and enalapril, have been implicated as holding importance within distinct pathways (including hypersensitivity) disconnected to pharmacological action.[50, 83, 84] It should be added that, owing to the presence of free carboxylic acid units, acyl glucuronide formation is an additional possibility.

3.3.5.3. Statin

Antilipidaemic therapeutics deriving efficacy from ability to mimic the native substrate of the HMG-CoA reductase enzyme, and defined structurally by possession of a seven-membered carbon chain (cyclic or linear) terminating in a beta-hydroxy carboxyl unit. Twelve compounds were found to trigger this alert, of which seven had definitive relationship with cholestatic outcome – including many of the most commonly-prescribed members amongst the family, such as simvastatin, atorvastatin, lovastatin and pravastatin. Disease is typically observed to follow a pattern consistent with idiosyncratic toxicity, with inflammation manifesting alongside impairment of bile motility.[85] Evidence for the involvement of cytochrome P450-derived reactive metabolites in the mediation of statin-induced hepatocellular injury has been forwarded – although the identity of such species remains undetermined.[86] Frequency of occurrence in patients is low, a factor which may explain the apparent absence of the effect within the remaining five entries – each of which are comparatively uncommon in clinical use (e.g. bervastatin, glenvastatin and dalvastatin).[50]

3.4. Further applications of cholestasis structural alerts

There are a variety of potential applications relating to the alerts described above. In part, they and their associated chemistry may be considered to define, at least initially, a domain for cholestasis which may function within the mode of action (MOA) ontology to permit linkages to other properties important for risk assessment.[87] It is apparent from the evidence provided through this study that distinct groupings of chemicals are present, which in turn may be expanded upon. Whilst the scope of

compounds examined was limited by necessity to pharmaceuticals, it should be stated that the alerts derived are in principle applicable to all chemicals, independent of use class. It is important, nevertheless, to appreciate the caveats associated with their adoption. Of the fifteen alerts reported, none exhibit exclusive selectivity for cholestasis. Reasons for this may well lie in the factors discussed within Section 3.1 – namely, the potential for occurrence of false-negatives arising through presence of compounds subject to comparatively infrequent clinical use. Such knowledge cannot be incorporated into the model at this time, but must instead be borne in mind by the user. This may be pertinent within applications including hazard assessment and read-across, where limitations associated with the alert and the data informing it must be considered.

The approach applied in this investigation – namely the primary use of expert knowledge supplemented through informatics approaches – has led to the production of a series of alerts which are both varied and robust. Such a method has significant advantages over the use of fragment-based technologies alone, in that the constructed alerts are both supported by experimental data and further provided with strong mechanistic underpinning. The latter point provides the linkage between mechanistic understanding and the chemistry domain for the effect, and as such is analogous to gaining information from the MIE. Whilst such knowledge may be challenging and time-consuming to compile, it nevertheless provides reductions in uncertainties which may be present inherently within *in silico* modelling approaches – helping in turn to justify the methodology and improve confidence in predictions obtained through it.[88, 89]

4. Conclusion

From a dataset composed of greater than 1500 (predominantly pharmaceutical) compounds, fifteen distinct structural alerts associated with emergence of clinical cholestatic liver injury have been developed. These span a variety of chemical and therapeutic classes, from steroidal and non-steroidal hormone receptor modulators to tricyclic psychoactives and antimicrobial sulfonamides and macrolides. Mechanistic insight is provided in each case, linking, where possible, defining structural

features with induction of disease. Within the majority of instances, this is apparently the result of idiosyncratic reaction arising as a consequence of metabolic or immunological abnormality – as such unrelated to intrinsic pharmacology. Accordingly, alerts generally describe substructures liable to undergo enzymatic activation to reactive intermediates (quinone-imines, epoxides, acyl glucuronides) and henceforth form adducts with proteins. It is acknowledged that, owing to the overrepresentation of cholestasis-negative compounds within the training set, the apparent selectivity of each alert may be understated. We posit that in numerous instances, practices related to the deployment of compounds in the clinical setting – be it for example, through general rarity of use – may influence reported occurrence of the endpoint. Informatics analysis largely supported the composition of the constructed alerts, with substantial overlap apparent between both them and the chemotype fragments most selective for cholestasis-positive compounds. Potential for utilisation of these alerts exists in fields ranging from hazard identification and prioritisation, to AOP rationalisation and assistance in read-across.

Associated Content

Supporting Information 1: containing all Supplementary Tables (xls).

Supplementary Table 1: List of compounds, sourced from Kotsampasakou and Ecker, composing full cholestasis dataset

Supplementary Table 2: Outcomes of ToxPrint chemotype analysis of cholestasis dataset

Acknowledgement

This work was financially supported by Cosmetics Europe as part of the Long Range Science Strategy programme and by the European Chemical Industry Council (CEFIC) as part of the project "Development and testing of a repeated dose ontology model for chemical risk assessment purposes: liver effects as a case study".

Declaration of Interest

The authors declare no conflicts of interest.

4. References

(1) Kaplowitz, N. (2013) Chapter 1 - Drug-Induced Liver Injury: Introduction and Overview, In *Drug-Induced Liver Disease (Third Edition)* (Kaplowitz, N., and DeLeve, L. D., Eds.) pp 3-14, Academic Press, Boston, USA.

(2) Béquignon, O. J., Pawar, G., van de Water, B., Cronin, M. T., and van Westen, G. J. (2019) Computational Approaches for Drug-Induced Liver Injury (DILI) Prediction: State of the Art and Challenges. *Reference Module in Biomedical Sciences*.

(3) Kullak-Ublick, G. (2013) Drug-induced cholestatic liver disease, In *Madame Curie bioscience database [Internet]*, Landes Bioscience.

(4) Padda, M. S., Sanchez, M., Akhtar, A. J., and Boyer, J. L. (2011) Drug-induced cholestasis. *Hepatology 53*, 1377-1387.

(5) Bhamidimarri, K. R., and Schiff, E. (2013) Drug-Induced Cholestasis. *Clinics in Liver Disease 17*, 519-531.

(6) Hamilton, J. P., and Laurin, J. M. (2008) Drug-Induced Cholestasis, In *Cholestatic Liver Disease* (Lindor, K. D., and Talwalkar, J. A., Eds.) pp 21-43, Humana Press, Totowa, NJ.

(7) Trauner, M., Meier, P. J., and Boyer, J. L. (1999) Molecular regulation of hepatocellular transport systems in cholestasis. *J Hepatol 31*, 165-178.

(8) Dawson, P. A., Lan, T., and Rao, A. (2009) Bile acid transporters. *J Lipid Res 50*, 2340-2357.

(9) Wagner, M., Zollner, G., and Trauner, M. (2009) New molecular insights into the mechanisms of cholestasis. *Journal of Hepatology 51*, 565-580.

(10) Garzel, B., Yang, H., Zhang, L., Huang, S.-M., Polli, J. E., and Wang, H. (2014) The role of bile salt export pump gene repression in drug-induced cholestatic liver toxicity. *Drug metabolism and disposition: the biological fate of chemicals 42*, 318-322.

(11) Uetrecht, J. (2019) Mechanistic Studies of Idiosyncratic DILI: Clinical Implications. *Frontiers in Pharmacology 10*.

(12) Uetrecht, J. (2013) Chapter 11 - Role of the Adaptive Immune System in Idiosyncratic Drug-Induced Liver Injury, In *Drug-Induced Liver Disease (Third Edition)* (Kaplowitz, N., and DeLeve, L. D., Eds.) pp 175-193, Academic Press, Boston, USA.

(13) Liu, Z. X., and Kaplowitz, N. (2002) Immune-mediated drug-induced liver disease. *Clin Liver Dis 6*, 755-774.

(14) Fontana, R. J. (2014) Pathogenesis of idiosyncratic drug-induced liver injury and clinical perspectives. *Gastroenterology 146*, 914-928.

(15) Pedersen, J. M., Matsson, P., Bergström, C. A., Hoogstraate, J., Norén, A., LeCluyse, E. L., and Artursson, P. (2013) Early identification of clinically relevant drug interactions with the human bile salt export pump (BSEP/ABCB11). *Toxicol Sci 136*, 328-343.

(16) Morgan, R. E., van Staden, C. J., Chen, Y., Kalyanaraman, N., Kalanzi, J., Dunn, R. T., 2nd, Afshari, C. A., and Hamadeh, H. K. (2013) A multifactorial approach to hepatobiliary transporter assessment enables improved therapeutic compound development. *Toxicol Sci 136*, 216-241.

(17) Köck, K., Ferslew, B. C., Netterberg, I., Yang, K., Urban, T. J., Swaan, P. W., Stewart, P. W., and Brouwer, K. L. (2014) Risk factors for development of cholestatic drug-induced liver injury: inhibition of hepatic basolateral bile acid transporters multidrug resistance-associated proteins 3 and 4. *Drug Metab Dispos 42*, 665-674.

(18) Chan, R., and Benet, L. Z. (2017) Measures of BSEP Inhibition In Vitro Are Not Useful Predictors of DILI. *Toxicological Sciences 162*, 499-508.

(19) Deferm, N., De Vocht, T., Qi, B., Van Brantegem, P., Gijbels, E., Vinken, M., de Witte, P., Bouillon, T., and Annaert, P. (2019) Current insights in the complexities underlying drug-induced cholestasis. *Crit Rev Toxicol 49*, 520-548.

(20) Przybylak, K. R., and Cronin, M. T. D. (2012) In silico models for drug-induced liver injury – current status. *Expert Opinion on Drug Metabolism & Toxicology 8*, 201-217.
(21) Kotsampasakou, E., and Ecker, G. F. (2017) Predicting Drug-Induced Cholestasis with the Help of Hepatic Transporters-An in Silico Modeling Approach. *J Chem Inf Model 57*, 608-615.
(22) Xi, L., Yao, J., Wei, Y., Wu, X. a., Yao, X., Liu, H., and Li, S. (2017) The in silico identification of human bile salt export pump (ABCB11) inhibitors associated with cholestatic drug-induced liver injury. *Molecular BioSystems 13*, 417-424.
(23) Shin, H. K., Kang, M.-G., Park, D., Park, T., and Yoon, S. (2020) Development of Prediction Models for Drug-Induced Cholestasis, Cirrhosis, Hepatitis, and Steatosis Based on Drug and Drug Metabolite Structures. *Frontiers in Pharmacology 11*.
(24) Yang, H., Lou, C., Li, W., Liu, G., and Tang, Y. (2020) Computational Approaches to Identify Structural Alerts and Their Applications in Environmental Toxicology and Drug Discovery. *Chemical Research in Toxicology 33*, 1312-1322.
(25) Limban, C., Nuţă, D. C., Chiriţă, C., Negreș, S., Arsene, A. L., Goumenou, M., Karakitsios, S. P., Tsatsakis, A. M., and Sarigiannis, D. A. (2018) The use of structural alerts to avoid the toxicity of pharmaceuticals. *Toxicology Reports 5*, 943-953.
(26) Hewitt, M., Enoch, S. J., Madden, J. C., Przybylak, K. R., and Cronin, M. T. (2013) Hepatotoxicity: a scheme for generating chemical categories for read-across, structural alerts and insights into mechanism(s) of action. *Crit Rev Toxicol 43*, 537-558.
(27) Liu, R., Yu, X., and Wallqvist, A. (2015) Data-driven identification of structural alerts for mitigating the risk of drug-induced human liver injuries. *J Cheminform 7*, 4.
(28) Mellor, C., Cronin, M., and Steinmetz, F. (2014) Identification of in silico structural alerts for liver steatosis induced by nuclear receptor agonists. *Toxicology Letters 229*, S162.
(29) Mellor, C. L., Steinmetz, F. P., and Cronin, M. T. D. (2016) Using Molecular Initiating Events to Develop a Structural Alert Based Screening Workflow for Nuclear Receptor Ligands Associated with Hepatic Steatosis. *Chemical Research in Toxicology 29*, 203-212.
(30) Yang, C., Tarkhov, A., Marusczyk, J., Bienfait, B., Gasteiger, J., Kleinoeder, T., Magdziarz, T., Sacher, O., Schwab, C. H., Schwoebel, J., Terfloth, L., Arvidson, K., Richard, A., Worth, A., and Rathman, J. (2015) New Publicly Available Chemical Query Language, CSRML, To Support Chemotype Representations for Application to Data Mining and Modeling. *Journal of Chemical Information and Modeling 55*, 510-528.
(31) Rathman J.F., Yang C., Ribeiro V., Mostrag A., Thakkar S., Tong W., Hobocienski B., Sacher O., Magdziarz T., Bienfait B. (2020) Development of A Battery of Prediction Tools for Drug-Induced Liver Injury (DILI) from the Vantage Point of Translational Safety Assessment. [Manuscript submitted for publication]
(32) Agresti, A. (2012) *Categorical Data Analysis (Third Edition)*. Wiley, Hoboken, USA
(33) Sprandel, K. A., and Rodvold, K. A. (2003) Safety and tolerability of fluoroquinolones. *Clin Cornerstone Suppl 3*, S29-36.
(34) De Liguoro, M., Fioretto, B., Poltronieri, C., and Gallina, G. (2009) The toxicity of sulfamethazine to Daphnia magna and its additivity to other veterinary sulfonamides and trimethoprim. *Chemosphere 75*, 1519-1524.
(35) Brock, N. (1994) Acepromazine revisited. *Can Vet J 35*, 458-459.
(36) Tačić, A., Nikolić, V., Nikolić, L., and Savić, I. (2017) Antimicrobial sulfonamide drugs. *Advanced Technologies 6*, 58-71.
(37) Leonardi, A., and Quintieri, L. (2010) Olopatadine: a drug for allergic conjunctivitis targeting the mast cell. *Expert Opinion on Pharmacotherapy 11*, 969-981.
(38) Song, X., Vasilenko, A., Chen, Y., Valanejad, L., Verma, R., Yan, B., and Deng, R. (2014) Transcriptional dynamics of bile salt export pump during pregnancy: mechanisms and implications in intrahepatic cholestasis of pregnancy. *Hepatology 60*, 1993-2007.

(39) Chen, Y., Vasilenko, A., Song, X., Valanejad, L., Verma, R., You, S., Yan, B., Shiffka, S., Hargreaves, L., Nadolny, C., and Deng, R. (2015) Estrogen and Estrogen Receptor-α-Mediated Transrepression of Bile Salt Export Pump. *Mol Endocrinol 29*, 613-626.
(40) Stolz, A., Navarro, V., Hayashi, P. H., Fontana, R. J., Barnhart, H. X., Gu, J., Chalasani, N. P., Vega, M. M., Bonkovsky, H. L., Seeff, L. B., Serrano, J., Avula, B., Khan, I. A., Cirulli, E. T., Kleiner, D. E., and Hoofnagle, J. H. (2019) Severe and protracted cholestasis in 44 young men taking bodybuilding supplements: assessment of genetic, clinical and chemical risk factors. *Aliment Pharmacol Ther 49*, 1195-1204.
(41) El Sherrif, Y., Potts, J. R., Howard, M. R., Barnardo, A., Cairns, S., Knisely, A. S., and Verma, S. (2013) Hepatotoxicity from anabolic androgenic steroids marketed as dietary supplements: contribution from ATP8B1/ABCB11 mutations? *Liver Int 33*, 1266-1270.
(42) Liebe, R., Krawczyk, M., Raszeja-Wyszomirska, J., Kruk, B., Preis, R., Trottier, J., Barbier, O., Milkiewicz, P., and Lammert, F. (2016) Heterozygous Inactivation of the Nuclear Receptor PXR/NR1I2 in a Patient With Anabolic Steroid-Induced Intrahepatic Cholestasis. *Hepat Mon 16*, e35953-e35953.
(43) Sanoh, S., Kitamura, S., Sugihara, K., Fujimoto, N., and Ohta, S. (2003) Estrogenic activity of stilbene derivatives. *J. Health Sci. 49*, 359-367.
(44) Garzel, B., Hu, T., Li, L., Lu, Y., Heyward, S., Polli, J., Zhang, L., Huang, S.-M., Raufman, J.-P., and Wang, H. (2020) Metformin Disrupts Bile Acid Efflux by Repressing Bile Salt Export Pump Expression. *Pharmaceutical Research 37*, 26.
(45) Walgren, J. L., Mitchell, M. D., and Thompson, D. C. (2005) Role of metabolism in drug-induced idiosyncratic hepatotoxicity. *Crit Rev Toxicol 35*, 325-361.
(46) Park, B. K., Kitteringham, N. R., Maggs, J. L., Pirmohamed, M., and Williams, D. P. (2005) The role of metabolic activation in drug-induced hepatotoxicity. *Annu Rev Pharmacol Toxicol 45*, 177-202.
(47) Dehal, S. S., and Kupfer, D. (1999) Cytochrome P-450 3A and 2D6 catalyze ortho hydroxylation of 4-hydroxytamoxifen and 3-hydroxytamoxifen (droloxifene) yielding tamoxifen catechol: involvement of catechols in covalent binding to hepatic proteins. *Drug Metab Dispos 27*, 681-688.
(48) Andrade, R. J., and Tulkens, P. M. (2011) Hepatic safety of antibiotics used in primary care. *J Antimicrob Chemother 66*, 1431-1446.
(49) Slatore, C. G., and Tilles, S. A. (2004) Sulfonamide hypersensitivity. *Immunol Allergy Clin North Am 24*, 477-490, vii.
(50) Halegoua-De Marzio, D., and Navarro, V. J. (2013) Chapter 29 - Hepatotoxicity of Cardiovascular and Antidiabetic Drugs, In *Drug-Induced Liver Disease (Third Edition)* (Kaplowitz, N., and DeLeve, L. D., Eds.) pp 519-540, Academic Press, Boston, USA.
(51) Welling, P. G. (1986) Pharmacokinetics of the thiazide diuretics. *Biopharm. Drug Dispos. 7*, 501-535.
(52) Brørs, O., and Jacobsen, S. (1979) Pharmacokinetics of hydroflumethiazide during repeated oral administration to healthy subjects. *European Journal of Clinical Pharmacology 15*, 281-286.
(53) Gold, B., and Mirvish, S. S. (1977) N-Nitroso derivatives of hydrochlorothiazide, niridazole, and tolbutamide. *Toxicology and Applied Pharmacology 40*, 131-136.
(54) Kalgutkar, A. S., Jones, R., and Sawant, A. (2010) Chapter 5 Sulfonamide as an Essential Functional Group in Drug Design, In *Metabolism, Pharmacokinetics and Toxicity of Functional Groups: Impact of Chemical Building Blocks on ADMET* pp 210-274, The Royal Society of Chemistry.
(55) Selim, K., and Kaplowitz, N. (1999) Hepatotoxicity of psychotropic drugs. *Hepatology 29*, 1347-1351.

(56) Wen, B., and Zhou, M. (2009) Metabolic activation of the phenothiazine antipsychotics chlorpromazine and thioridazine to electrophilic iminoquinone species in human liver microsomes and recombinant P450s. *Chem Biol Interact 181*, 220-226.
(57) MacAllister, S. L., Young, C., Guzdek, A., Zhidkov, N., and O'Brien, P. J. (2013) Molecular cytotoxic mechanisms of chlorpromazine in isolated rat hepatocytes. *Canadian Journal of Physiology and Pharmacology 91*, 56-63.
(58) Wen, B., Ma, L., and Zhu, M. (2008) Bioactivation of the tricyclic antidepressant amitriptyline and its metabolite nortriptyline to arene oxide intermediates in human liver microsomes and recombinant P450s. *Chemico-Biological Interactions 173*, 59-67.
(59) Milionis, H. J., Skopelitou, A., and Elisaf, M. S. (2000) Hypersensitivity syndrome caused by amitriptyline administration. *Postgraduate Medical Journal 76*, 361-363.
(60) Dekker, S. J., Zhang, Y., Vos, J. C., Vermeulen, N. P., and Commandeur, J. N. (2016) Different Reactive Metabolites of Nevirapine Require Distinct Glutathione S-Transferase Isoforms for Bioinactivation. *Chem Res Toxicol 29*, 2136-2144.
(61) Bu, H. Z., Kang, P., Deese, A. J., Zhao, P., and Pool, W. F. (2005) Human in vitro glutathionyl and protein adducts of carbamazepine-10,11-epoxide, a stable and pharmacologically active metabolite of carbamazepine. *Drug Metab Dispos 33*, 1920-1924.
(62) Gan, J., Zhang, H., and Humphreys, W. G. (2016) Drug-Protein Adducts: Chemistry, Mechanisms of Toxicity, and Methods of Characterization. *Chem Res Toxicol 29*, 2040-2057.
(63) Moseley, R. H. (2013) Chapter 26 - Hepatotoxicity of Antimicrobials and Antifungal Agents, In *Drug-Induced Liver Disease (Third Edition)* (Kaplowitz, N., and DeLeve, L. D., Eds.) pp 463-481, Academic Press, Boston, USA.
(64) Ariza, A., Mayorga, C., Fernandez, T. D., Barbero, N., Martín-Serrano, A., Pérez-Sala, D., Sánchez-Gómez, F. J., Blanca, M., Torres, M. J., and Montanez, M. I. (2015) Hypersensitivity reactions to β-lactams: relevance of hapten-protein conjugates. *J Investig Allergol Clin Immunol 25*, 12-25.
(65) Weltzien, H. U., and Padovan, E. (1998) Molecular Features of Penicillin Allergy. *Journal of Investigative Dermatology 110*, 203-206.
(66) Ariza, A., Collado, D., Vida, Y., Montañez, M. I., Pérez-Inestrosa, E., Blanca, M., Torres, M. J., Cañada, F. J., and Pérez-Sala, D. (2014) Study of protein haptenation by amoxicillin through the use of a biotinylated antibiotic. *PLoS One 9*, e90891.
(67) Carey, M. A., and van Pelt, F. N. A. M. (2005) Immunochemical detection of flucloxacillin adduct formation in livers of treated rats. *Toxicology 216*, 41-48.
(68) Hasdenteufel, F., Luyasu, S., Hougardy, N., Fisher, M., Boisbrun, M., Mertes, P. M., and Kanny, G. (2012) Structure-activity relationships and drug allergy. *Curr Clin Pharmacol 7*, 15-27.
(69) Pessayre, D., Larrey, D., Funck-Brentano, C., and Benhamou, J. P. (1985) Drug interactions and hepatitis produced by some macrolide antibiotics. *J Antimicrob Chemother 16 Suppl A*, 181-194.
(70) Castagnola, E., Machetti, M., Bucci, B., and Viscoli, C. (2004) Antifungal prophylaxis with azole derivatives. *Clin Microbiol Infect 10 Suppl 1*, 86-95.
(71) Orman, E. S., Conjeevaram, H. S., Vuppalanchi, R., Freston, J. W., Rochon, J., Kleiner, D. E., Hayashi, P. H., and Group, D. R. (2011) Clinical and histopathologic features of fluoroquinolone-induced liver injury. *Clin Gastroenterol Hepatol 9*, 517-523.e513.
(72) Schmid, D. A., Campi, P., and Pichler, W. J. (2006) Hypersensitivity reactions to quinolones. *Curr Pharm Des 12*, 3313-3326.
(73) Pichler, W. J. (2003) Delayed drug hypersensitivity reactions. *Ann Intern Med 139*, 683-693.
(74) Pichler, W. J. (2008) The p-i Concept: Pharmacological Interaction of Drugs With Immune Receptors. *World Allergy Organ J 1*, 96-102.
(75) Fung, M., Thornton, A., Mybeck, K., Wu, J. H.-h., Hornbuckle, K., and Muniz, E. (2001) Evaluation of the Characteristics of Safety Withdrawal of Prescription Drugs from Worldwide Pharmaceutical Markets-1960 to 1999. *Drug Information Journal 35*, 293-317.

(76) Trechot, P., Gillet, P., Gay, G., Hanesse, B., Netter, P., Castot, A., and Larrey, D. (1996) Incidence of hepatitis induced by non-steroidal anti-inflammatory drugs (NSAID). *Ann Rheum Dis 55*, 936.
(77) Delgado, C., Bentley, E., Hetzel, S., and Smith, L. J. (2014) Comparison of carprofen and tramadol for postoperative analgesia in dogs undergoing enucleation. *J Am Vet Med Assoc 245*, 1375-1381.
(78) Boelsterli, U. A. (2013) Chapter 21 - Mechanisms Underlying the Hepatotoxicity of Nonsteroidal Antiinflammatory Drugs, In *Drug-Induced Liver Disease (Third Edition)* (Kaplowitz, N., and DeLeve, L. D., Eds.) pp 343-367, Academic Press, Boston, USA.
(79) Wade, L. T., Kenna, J. G., and Caldwell, J. (1997) Immunochemical identification of mouse hepatic protein adducts derived from the nonsteroidal anti-inflammatory drugs diclofenac, sulindac, and ibuprofen. *Chem Res Toxicol 10*, 546-555.
(80) Horng, H., Spahn-Langguth, H., and Benet, L. Z. (2013) Chapter 3 - Mechanistic Role of Acyl Glucuronides, In *Drug-Induced Liver Disease (Third Edition)* (Kaplowitz, N., and DeLeve, L. D., Eds.) pp 35-70, Academic Press, Boston, USA.
(81) Darnell, M., Breitholtz, K., Isin, E. M., Jurva, U. and Weidolf L. (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
(82) Douros, A., Kauffmann, W., Bronder, E., Klimpel, A., Garbe, E., and Kreutz, R. (2013) Ramipril-Induced Liver Injury: Case Report and Review of the Literature. *American Journal of Hypertension 26*, 1070-1075.
(83) Rahmat, J., Gelfand, R. L., Gelfand, M. C., Winchester, J. F., Schreiner, G. E., and Zimmerman, H. J. (1985) Captopril-Associated Cholestatic Jaundice. *Annals of Internal Medicine 102*, 56-58.
(84) Singh, G., Kachalia, A., Kaur, J., Kachalia, K., Liu, S., and Rizzo, V. (2014) Cholestatic hepatitis: an unusual presentation of lisinopril induced hepatotoxicity. *British Journal of Medical Practitioners 7*, 34+.
(85) Björnsson, E., Jacobsen, E. I., and Kalaitzakis, E. (2012) Hepatotoxicity associated with statins: reports of idiosyncratic liver injury post-marketing. *J Hepatol 56*, 374-380.
(86) Abdoli, N., Heidari, R., Azarmi, Y., and Eghbal, M. A. (2013) Mechanisms of the statins cytotoxicity in freshly isolated rat hepatocytes. *J Biochem Mol Toxicol 27*, 287-294.
(87) Desprez, B., Birk, B., Blaauboer, B., Boobis, A., Carmichael, P., Cronin, M. T. D., Curie, R., Daston, G., Hubesch, B., Jennings, P., Klaric, M., Kroese, D., Mahony, C., Ouédraogo, G., Piersma, A., Richarz, A.-N., Schwarz, M., van Benthem, J., van de Water, B., and Vinken, M. (2019) A mode-of-action ontology model for safety evaluation of chemicals: Outcome of a series of workshops on repeated dose toxicity. *Toxicology in Vitro 59*, 44-50.
(88) Schultz, T. W., Richarz, A.-N., and Cronin, M. T. D. (2019) Assessing uncertainty in read-across: Questions to evaluate toxicity predictions based on knowledge gained from case studies. *Computational Toxicology 9*, 1-11.
(89) Cronin, M. T. D., Richarz, A.-N., and Schultz, T. W. (2019) Identification and description of the uncertainty, variability, bias and influence in quantitative structure-activity relationships (QSARs) for toxicity prediction. *Regulatory Toxicology and Pharmacology 106*, 90-104.