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

3

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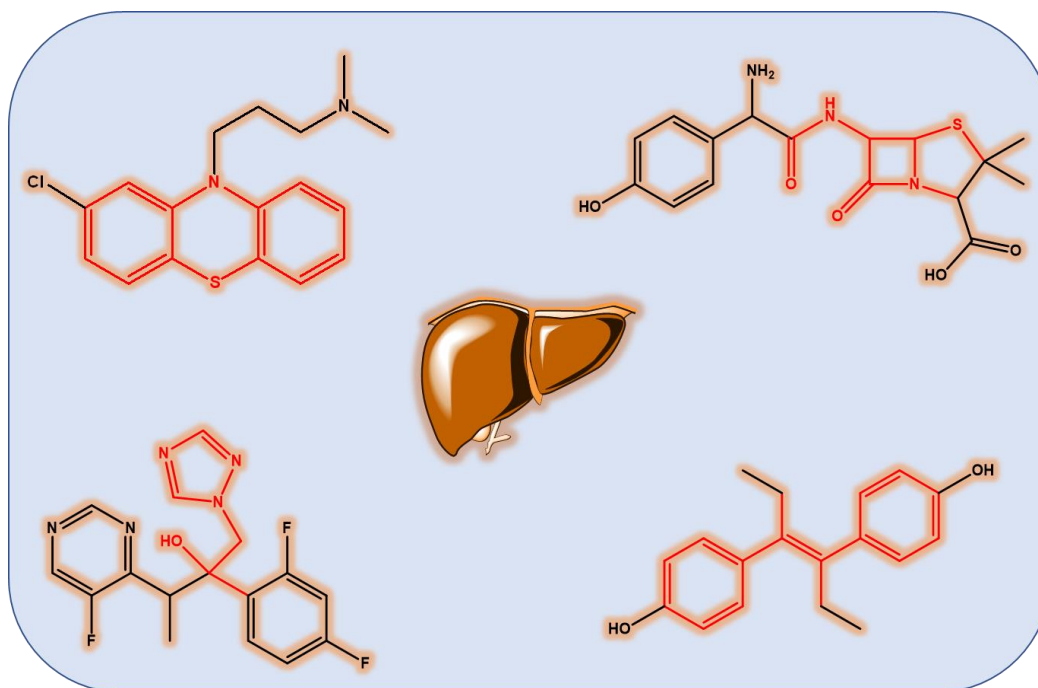
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17



18

19 *Graphic for table of contents use*

20

21

22 **Abstract**

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

41

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

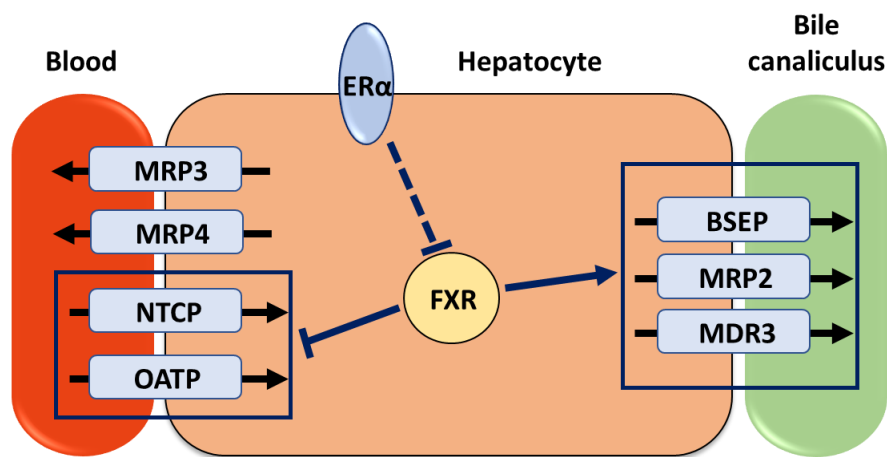
43 **1. Introduction**

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

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

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

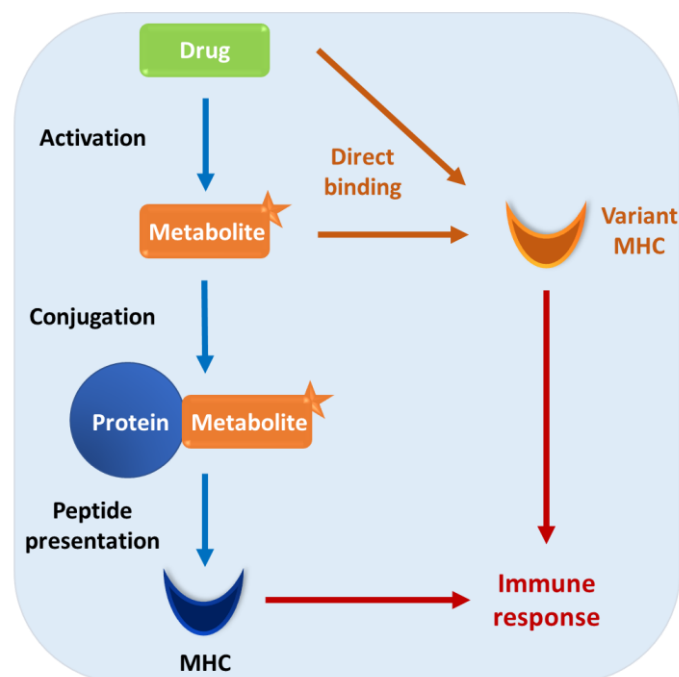
68 of the organic anion transporting polypeptide (OATP) class.^[7] Secretion of bile salts, conjugated
 69 bilirubin and minor fatty acid substituents into the bile canaliculi is mediated by a still greater number
 70 of such transporters, of which the most prominent are those belonging to the of the ATP-binding
 71 cassette (ABC) family. Amongst this number are the bile salt export pump (BSEP) and multi-drug
 72 resistance-associated protein 2 (MRP2).^[8] A complex regulatory system, incorporating (amongst other
 73 influences) the oestrogen (ER) and farnesoid X receptors (FXR), mediates functioning of this network.^[9]
 74 ^{10]} Oestrogenic and androgenic steroids, alongside their derivatives, are most commonly linked with
 75 the emergence of this form of disease.



76
 77 **Figure 1.** Overview of systems integral within intrahepatic transport of bile components. Uptake from portal
 78 blood is mediated by OATP and NTCP transporters, whilst extrusion into bile canaliculi occurs through the action
 79 of proteins of the ABC family (most prominently BSEP). Both processes are under regulation of the FXR – the
 80 former negatively, the latter positively – with the net impact of maintaining hepatocellular bile concentration at
 81 physiological level. Hypothesised repression of FXR owing to oestrogen receptor cross-talk is depicted, as is the
 82 presence of two further transporters (MRP3 and MRP4) held to assist in transferral of bile substituents to the
 83 bloodstream.

84
 85 By contrast, the mechanisms underpinning emergence of mixed cholestatic-hepatitis are known less
 86 definitively. In numerous instances, evidence points to the relevance of idiosyncratic toxicity –
 87 typically associated with genetic variants controlling metabolite generation or immune response.^[11]
 88 ^{12]} A generalised pathway for idiosyncratic immuno-allergic reaction, as depicted within Figure 2,
 89 would centre first upon the formation by hepatic enzymes (commonly of the cytochrome P450 family)
 90 of reactive drug metabolites. Inability to adequately detoxify these species permits their adduction of

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



103
104 **Figure 2.** Depiction of proposed mechanisms through which hepatic drug hypersensitivity response may be
105 initiated. Metabolic activation (generally enzymatic) of parent compound to yield reactive species functions as
106 an essential first step. These may proceed to form protein conjugates, which in turn hold capacity trigger
107 immune response following presentation on MHC complexes. Alternatively, either metabolites or parent
108 compound may bind directly at variant MHC or T-cell receptor isoforms, in turn influencing T-cell recruitment
109 and immune activity (“p-l” hypothesis).

110

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

120 *In silico*, or computational, modelling presents a range of options as regards the addressing of liver
121 toxicity – including either the direct prediction of adverse effect, prioritisation of compounds for
122 further testing or alternatively as contribution towards a scientific “weight of evidence” judgment.
123 The techniques that may be applied range from the use of read-across, through quantitative structure-
124 activity relationships (QSARs) and machine learning.^[2] Various factors contribute to ensuring that the
125 structure-based prediction of cholestasis remains a complex endeavour – not least the lack of
126 standardised assays and paucity of accessible robust databases relating to the endpoint, compounded
127 by the current incomplete understanding of mechanisms of action underlying it.^[20] As such, relatively
128 few *in silico* models focused solely upon cholestasis have been created.^[21-23] A notably powerful tool
129 within computational toxicology is the use of chemical structural alerts – a particular advantage being
130 that alerts can often be understood directly in the context of the molecular initiating event (MIE).^{[24,}
131 ^{25]} Not only may the MIE be employed in informing and validating the alert, but additional scope exists
132 for drawing of support from new approach methodology (NAM) data. Whilst there has accordingly
133 been a rich history of their use in various aspects relating to liver toxicity (both general and specific),
134 a robust series of alerts for cholestasis has yet to emerge.^[26-29]

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

141

142 **2. Materials and methods**

143 **2.1. Curation of data**

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

154

155 **2.2. Development of structural alerts**

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

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

161

162 **2.3. ToxPrint chemotype analysis**

163 ToxPrint chemotypes were generated using the publicly-available ChemoTyper application (version
164 1.0; Molecular Networks, Erlangen, Germany) in order to detect building blocks which may be suitable
165 in forming the basis of structural alerts. Selected chemotypes were drawn from those initially reported
166 within Yang et al., and further expanded by Rathman et al.^[30, 31] Selectivity with respect to occurrence
167 in cholestasis-positive compounds was quantified through determination of Z-score, as derived in
168 accordance with protocols described previously.^[24, 31] Identical analysis was performed upon a
169 selection of 305 marketed pharmaceuticals positive for generalised DILI, sourced from Rathman et al.
170 and referred to henceforth as the “human DILI” set. ^[31]

171

172 **2.4. Quantification of structural alert performance and selectivity**

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

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

176

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

180

$$181 \quad \text{Odds ratio} = \frac{TP * TN}{FP * FN} \quad eq (1)$$

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

183

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

184
185

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

186

187 **3. Results and discussion**

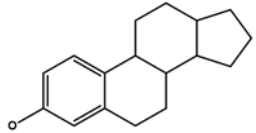
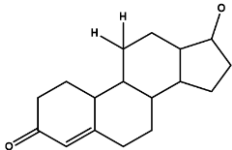
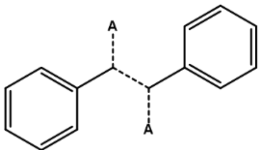
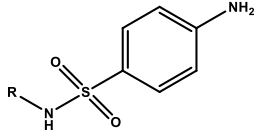
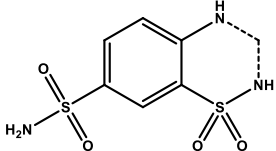
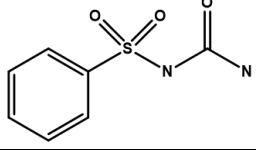
188 **3.1. Overview of compiled structural alerts**

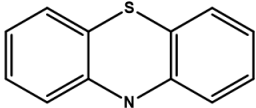
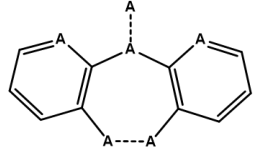
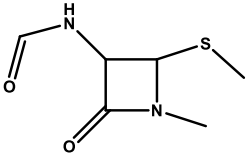
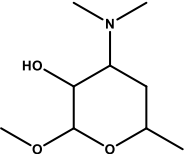
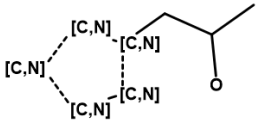
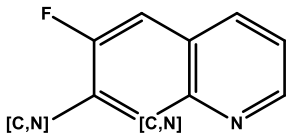
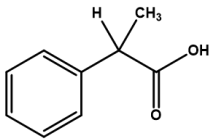
189 Fifteen structural alerts related to onset of clinical cholestasis were constructed and are depicted in
190 Table 2. Accompanying them are data concerning coverage and apparent selectivity – the latter
191 expressed in the form of odds ratios and positive predictivity values, as determined through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

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

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

215 Discussion related to the mechanistic and structure-activity aspects present within each category is
216 provided within Section 3.3 (refer to column titled “Postulated MIE” within Table 2 for overview). Data
217 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, haptentation (post-activation) | 4 | 2 | 7.40 | 0.67 |
| Sulfonamide | | | | | | |
| Sulfonamide (antimicrobial) |  | Protein nitrosylation, haptentation (post-activation) | 4 | 18 | 0.81 | 0.18 |
| Thiazide |  | Protein nitrosylation, haptentation (post-activation) | 17 | 2 | 32.73 | 0.89 |
| Benzene-sulfonylurea |  | Protein acylation, haptentation (post-activation) | 9 | 2 | 16.90 | 0.82 |
| Psychoactive tricyclic | | | | | | |

| | | | | | | |
|--------------------------|---|---|----|----|-------|------|
| Phenothiazine |  | Protein alkylation, haptentation (post-activation) | 16 | 7 | 8.74 | 0.70 |
| Dibenzo- cycloheptane |  | Protein alkylation, Haptentation (post-activation) | 19 | 6 | 12.23 | 0.76 |
| Anti-infective | | | | | | |
| Beta-lactam |  | Protein acylation, haptentation | 29 | 21 | 5.44 | 0.58 |
| Desosamine |  | Protein nitrosylation, haptentation (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, haptentation (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 |

218

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

221 3.2. Analysis of fragment selectivity through use of ToxPrint chemotypes

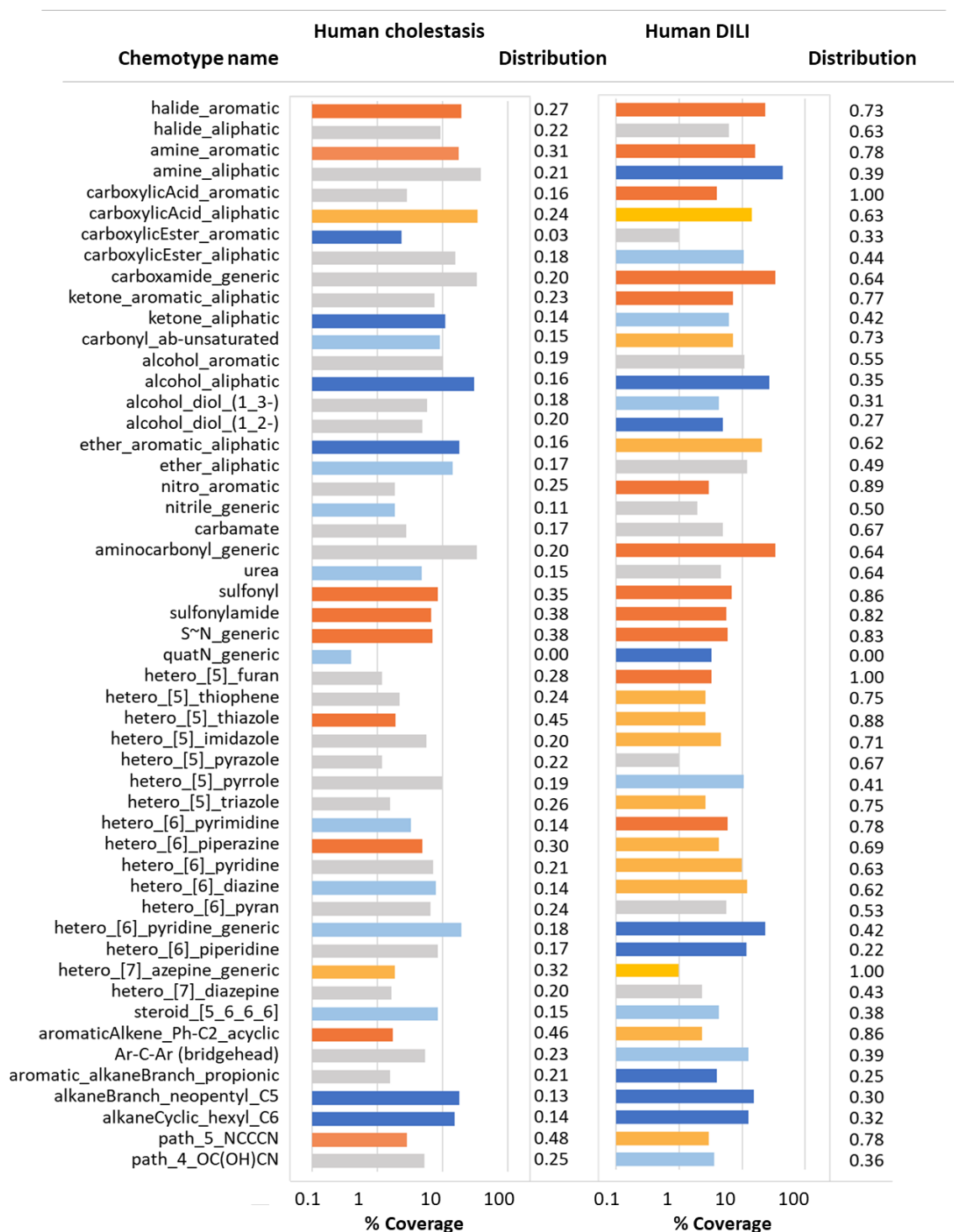
222 Preliminary profiling of the dataset using ToxPrint chemotypes revealed the identities of chemical
223 fragments displaying greatest frequency within (and selectivity for) cholestasis-positive compounds.
224 Listed within Table 3 are the chemotypes most reliably represented amongst this set, ordered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

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

232
233 **Table 3.** ToxPrint chemotypes present with greatest selectivity within cholestasis-positive compounds (ranked
234 according to Z-score). Distribution metric describes the proportion of compounds containing a given alert that
235 are also positive for cholestasis

236
237 Chemotype distribution amongst the cholestasis dataset was subsequently compared against that
238 within a further selection of pharmaceuticals judged for their capacity to induce generalised
239 hepatotoxicity (the “human DILI” set). Outcomes are depicted within Figure 3. It is apparent that, as

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



245

246 **Figure 3.** ToxPrint chemotype analysis of cholestasis and general DILI datasets. Bars are colour-coded in
247 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
248 dark blue ($Z \leq -2$). Length represents frequency of matches (% of structures in dataset containing given
249 chemotype), whilst the Distribution metric describes the proportion of compounds containing a given alert that
250 are also positive for cholestasis

251

252 **3.3. Alert descriptions**

253 **3.3.1. Steroid receptor modulator**

254 **3.3.1.1. Oestrogenic steroid**

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

268

269 **3.3.1.2. Androgenic steroid**

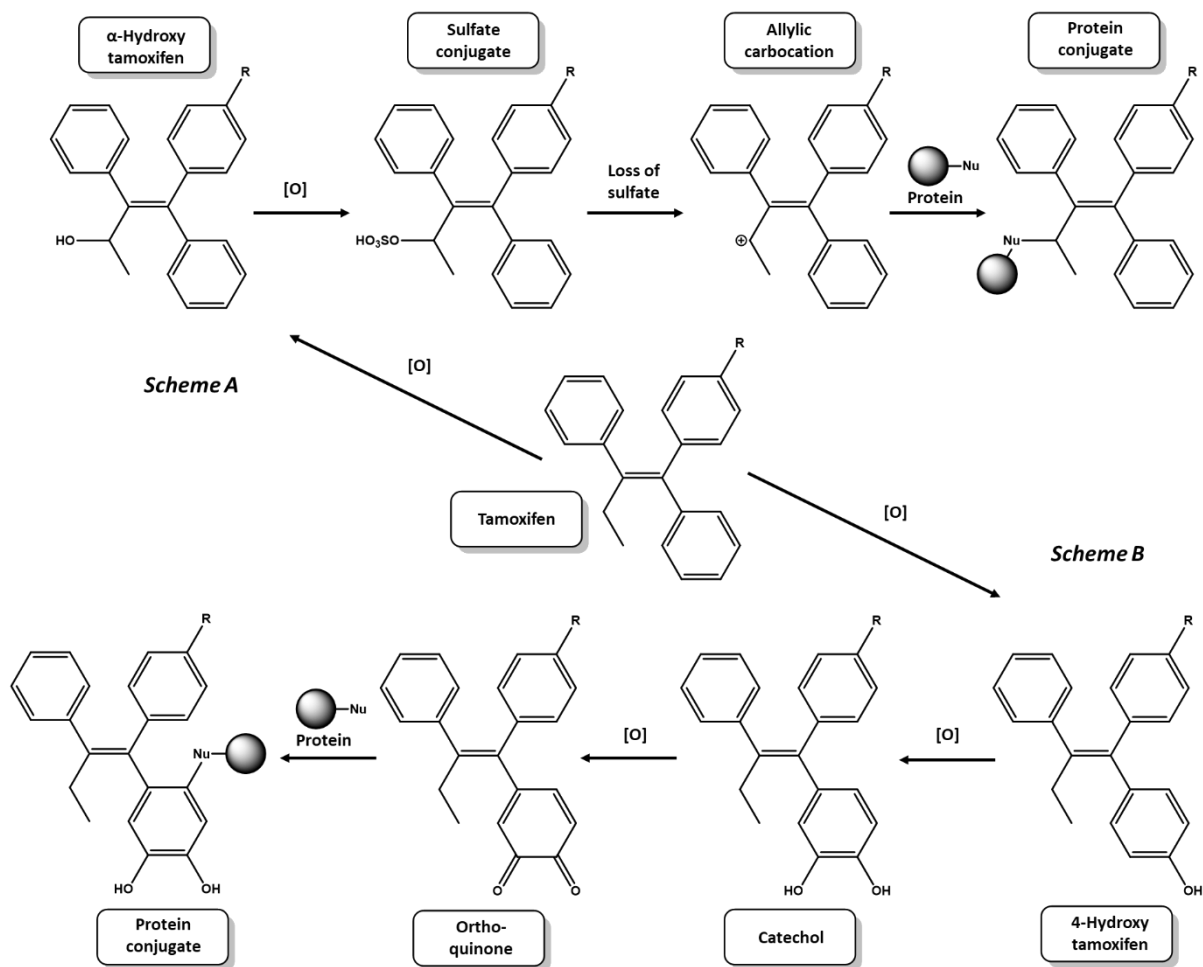
270 In a similar manner to their aforementioned oestrogenic counterparts, androgenic steroids deployed
271 for medicinal purpose have been observed to induce a form of bland cholestasis within susceptible
272 patients.^[40] The pathway underlying is largely undefined, although course of presentation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

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

279

280 **3.3.1.3. Stilbenoid**

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

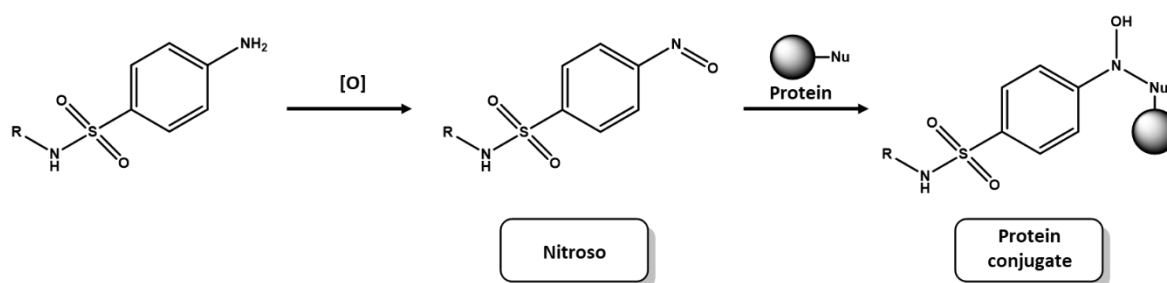
303

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

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

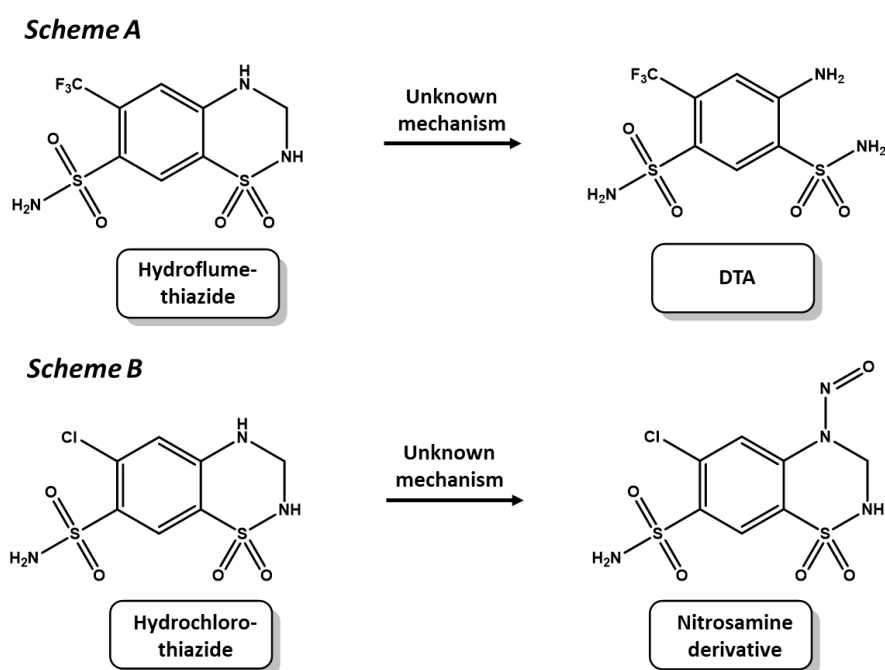


321
322 **Figure 5.** Oxidation of amino moiety positioned para- to sulfonamide unit, producing reactive nitroso metabolite.

323 324 3.3.2.2. Thiazide

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

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



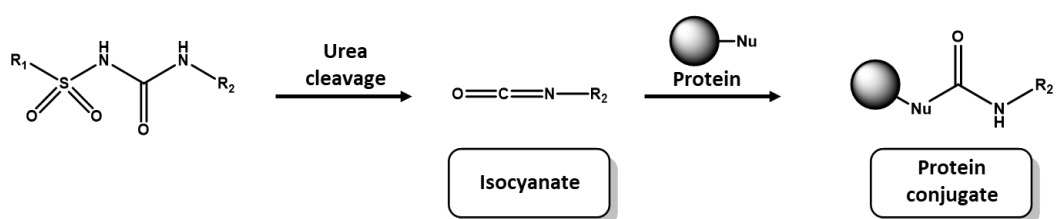
345 **Figure 6.** Occurring through an uncharacterised mechanism, transformation of hydroflumethiazide yielding 2,4-
346 disulfamyl-5-trifluoromethylaniline (DTA) – a para-amino sulfonamide – has been reported in man (Scheme A).
347 Depicted within Scheme B is the generation, *in chemico*, of a hydrochlorothiazide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

348

349 3.3.2.3. Benzenesulfonylurea

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

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



360
361 **Figure 7.** Cleavage of parent molecule about urea, occurring through an undefined mechanism, yielding
362 potentially reactive isocyanate.

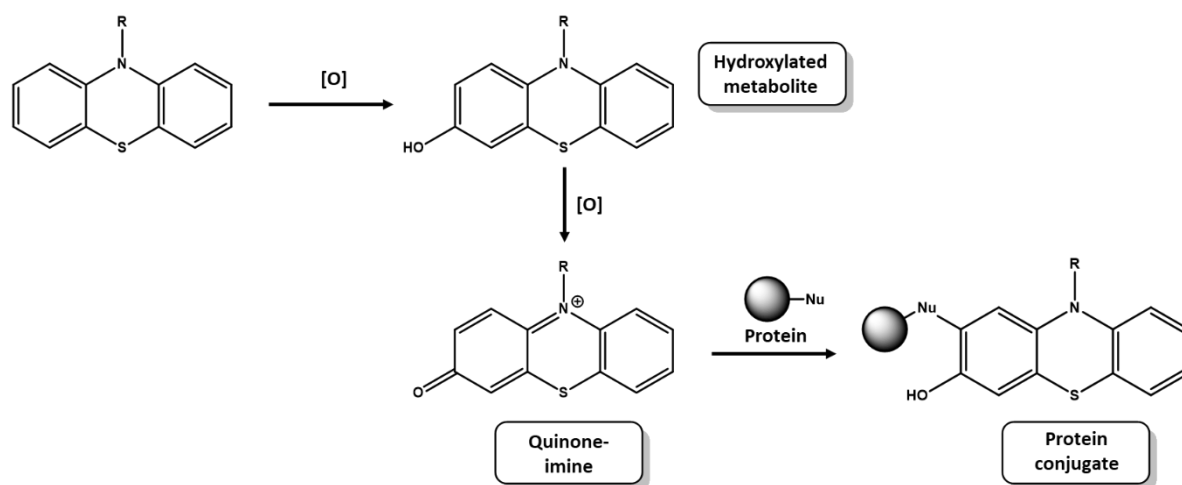
363

364 3.3.3. Psychoactive tricyclic

365 3.3.3.1. Phenothiazine

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

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



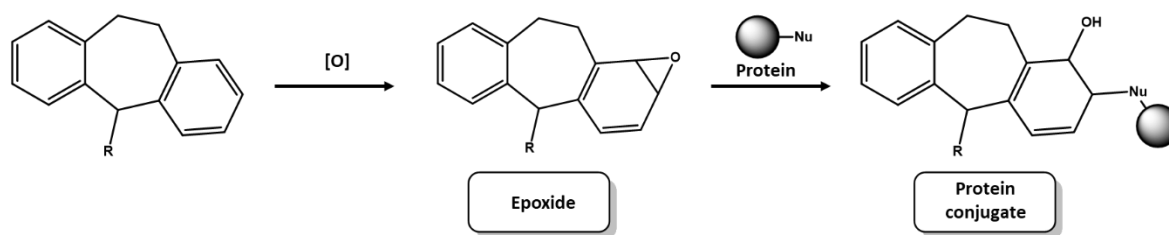
380 **Figure 8.** Scheme outlining formation of reactive quinone-imine intermediate following two-step oxidative
381 metabolism of phenothiazine core.

382

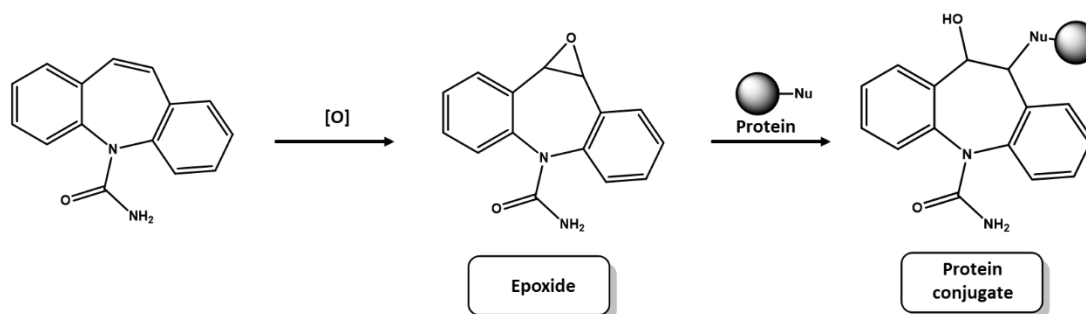
383 3.3.3.2. Dibenzocycloheptane

384 Like those amongst the phenothiazine class, dibenzocycloheptanes are capable of modulating activity
385 at neurotransmitter and histaminergic receptors – albeit with an effect profile which tends more
386 towards antidepressant than antipsychotic. A modified equivalent of this motif is further found within
387 the antiretroviral nevirapine. The nature of the alert allows for variation in composition amongst the
388 rings, and in all a total of 25 compounds are found bearing a form of it: nineteen associated with
389 cholestasis (idiosyncratic presentation), six not.^[55] In this instance, the enzymatic oxidation of
390 aromatic groups to yield labile epoxides is forwarded as a primary route through which activation of
391 the molecule might occur. Such a biotransformation has been noted to occur both in the
392 antidepressants amitriptyline and nortriptyline, and also in nevirapine (Figure 9, Scheme A).^[58-60] An
393 additional locus for epoxidation has been identified in compounds typified by carbamazepine, which
394 incorporate an alkene functionality within the central seven-membered ring (Figure 9, Scheme B).^{[61,}
395 ^{62]} Irrespective of the ultimate site of epoxide formation, apparent capacity to alkylate protein residues
396 remains conserved.

Scheme A



Scheme B



397

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

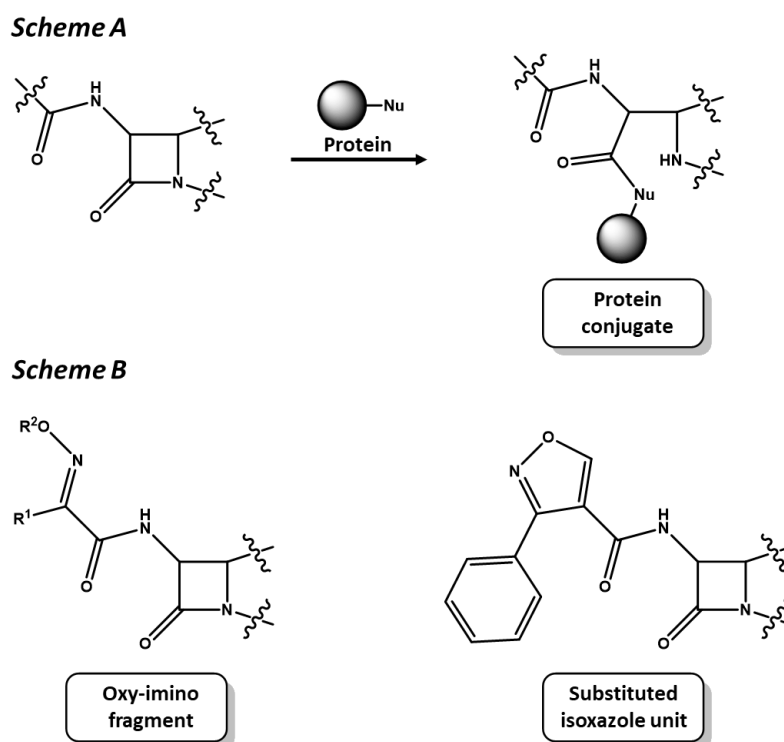
401

402 3.3.4. Anti-infective

403 3.3.4.1. Beta-lactam

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

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

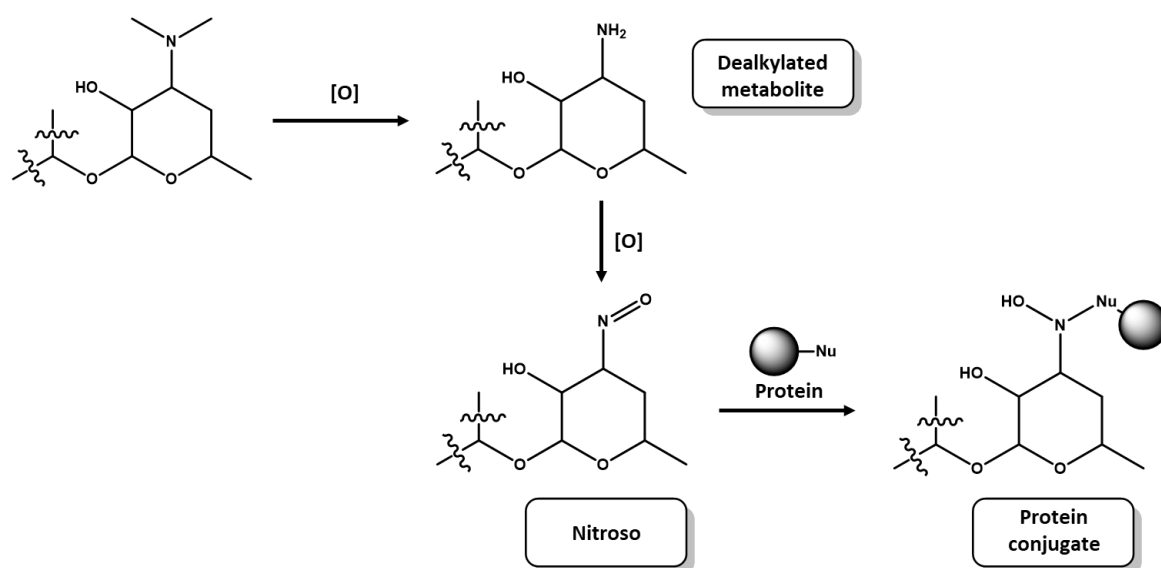


426
427 **Figure 10.** Opening of the intrinsically strained azetidinone ring through nucleophilic addition (Scheme A).
428 Depictions of side-chain features most reliably associated with emergence of cholestasis (Scheme B).

429 430 3.3.4.2. Desosamine

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

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



441
442 **Figure 11.** Two-stage oxidative metabolism of desosamine yielding reactive nitroso derivative.

443 444 **3.3.4.3. Azole antifungal**

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

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

455

456 **3.3.4.4. Fluoroquinolone**

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

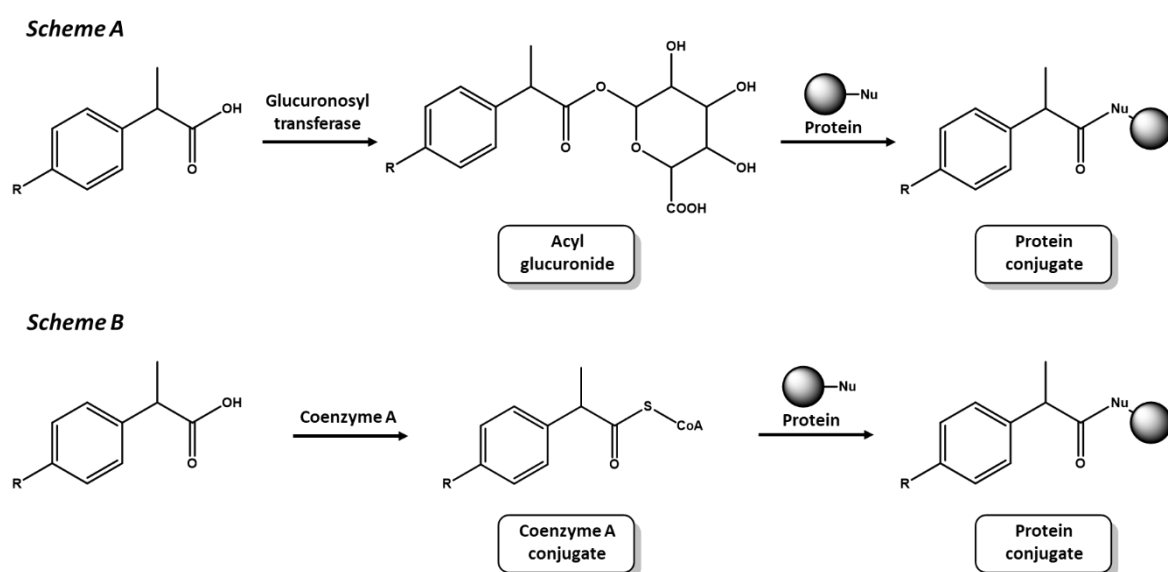
469

470 **3.3.5. Other**

471 **3.3.5.1. NSAID (-profen)**

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

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



485
 486 **Figure 12.** Metabolic transformation of carboxylic acid unit yielding potentially reactive acyl glucuronide
 487 (Scheme A) or coenzyme A (Scheme B) functionalities.

488
 489 **3.3.5.2. ACE inhibitor (peptidic)**

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

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

503

504 **3.3.5.3. Statin**

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

517

518 **3.4. Further applications of cholestasis structural alerts**

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

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

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

543

544 **4. Conclusion**

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

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

563

564 **Associated Content**

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

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

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

569

570 **Acknowledgement**

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572 programme and by the European Chemical Industry Council (CEFIC) as part of the project
573 “Development and testing of a repeated dose ontology model for chemical risk assessment purposes:
574 liver effects as a case study”.

575 **Declaration of Interest**

576 The authors declare no conflicts of interest.

577

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