



*Which are the most important reactions and enzymes in drug metabolism?
The answer depends on what 'important' means, but a meta-analysis
unveils some surprising figures.*

Foundation review: Reactions and enzymes in the metabolism of drugs and other xenobiotics

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In this article, we offer an overview of the compared quantitative importance of biotransformation reactions in the metabolism of drugs and other xenobiotics, based on a meta-analysis of current research interests. Also, we assess the relative significance the enzyme (super)families or categories catalysing these reactions. We put the facts unveiled by the analysis into a drug discovery context and draw some implications. The results confirm the primary role of cytochrome P450-catalysed oxidations and UDP-glucuronosyl-catalysed glucuronidations, but they also document the marked significance of several other reactions. Thus, there is a need for several drug discovery scientists to better grasp the variety of drug metabolism reactions and enzymes and their consequences.

Drug metabolism as a field of research was born during the first half of the 19th century, when hippuric acid (the glycine conjugate of benzoic acid) was discovered in horse urine (hence its name). It was only during the 1950s that drug metabolism really took off owing to a convergence of factors including (i) the progressive awareness among pharmaceutical scientists of the variety and significance of metabolic reactions and the involvement of metabolites in unwanted drug effects; (ii) the groundbreaking studies of distinguished pioneers; (iii) the explosive development of analytic instrumentation; and (iv) the acknowledged scientific and didactic impact of a few books. The landmark book of Williams, 'Detoxication Mechanisms' [1] was of special significance, particularly its much expanded 1959 second edition [2]. Since then, the study of the metabolism of drugs and other xenobiotics has become a multidisciplinary science combining chemistry (analytical, physical, organic, organometallic, structural, among others), biology (biochemistry, enzymology, genetics, among others), disposition (including absorption, distribution, accumulation, excretion and pharmacokinetics), pharmacology and toxicology (molecular and clinical), and environmental sciences.

Ironically, Williams himself was displeased with the title of his book, being well aware that metabolism could in many cases lead to an increase in toxicity (toxication) [3]. And indeed, one of the reasons for the vast number of papers on xenobiotic metabolism appearing in the literature is a serious concern for the toxicological and pharmacological consequences of metabolite formation [4–6]. Several drugs do produce metabolites whose pharmacological activity complements and/or

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prolongs their own [6–8], not to mention prodrugs designed to be intrinsically inactive and predictably transformed into a metabolite accounting for the full therapeutic activity of the parent compound [9,10]. A major cause of concern in drug discovery and development is the formation of toxic and/or chemically reactive (and hence potentially toxic) metabolites [11–20]. Such metabolites need to be detected as early as possible during the discovery and development phases because they represent a damaging cause of withdrawal during clinical trials and post-marketing [21–26].

Numerous studies have been directed towards identifying metabolism-caused hazards [27–29], with computational approaches being increasingly valued as front-line screening tools [30–36]. Globally, two broad strategies exist among algorithms that predict xenobiotic metabolism, namely specific ('local') and comprehensive ('global') methods, as discussed in the concluding remarks section. Thus, the production of regioselective metabolites (e.g. hydroxylation of an aryl versus an alkyl group) is predictable with the former methods, but the contribution of different pathways (e.g. oxidation versus glucuronidation) is not and requires global algorithms that encompass all or most metabolic reactions. In other words, all available specific algorithms are restricted to one or a few reaction types, hardly a broad enough net to 'catch' potentially reactive metabolites.

An informed view of the relative importance of drug-metabolising reactions and enzymes, be it in quantitative or qualitative (bioactivities) terms, is still missing. Such an assessment could draw attention to lesser studied enzyme systems and would help broaden the understanding of medicinal chemists. In a recent commentary [37], one of us (Testa) addressed the above gap in our knowledge and proposed a 'guesstimate', assigning 30% and

20% of all known metabolites to redox reactions catalysed by cytochrome P450 (CYP) and other oxidoreductases, respectively, 10% to reactions of hydrolysis and hydration, 20% to glucuronidations, 10% to sulphonations, 5% to glutathione-mediated reactions, and 5% to conjugations catalysed by other transferases or ligases. But as stated, this was but a guesstimate based on 40 years of experience as a researcher and teacher in drug metabolism. To approach a realistic picture, we undertook a meta-analysis of the recent primary literature by analysing the metabolic fate of over one thousand different substrates.

The primary objective of this meta-analysis was to offer an overview of the compared quantitative importance of biotransformation reactions in the metabolism of medicinal compounds and other xenobiotics, based on current research interests as reflected in the specialised literature. A second objective was to assess the relative significance of the enzyme (super)families or groups of enzymes catalysing these reactions. Specifically, our objective was not to assess the proportion of xenobiotics being substrates of the different drug-metabolising enzymes, but to obtain a quantitative estimate of the proportion of metabolites produced by such enzymes. In the remainder of this article, we summarise our approach and criteria before detailing the results, interpreting them and ultimately drawing some implications in a drug discovery context.

Collecting and analysing the literature

Rules

Our first objective was to classify and count the metabolic reactions of over 1000 different substrates, as published in three selected peer-reviewed primary journals during the years 2004–2009. The studies

TABLE 1

Criteria and rules of the meta-analysis

Search and selection criteria

Objective	To classify and count the metabolic reactions of 1000 different substrates.
Method	A systematic paper-by-paper search of metabolic studies was carried out in the primary literature.
Literature	Three major refereed research journals: <i>Chem. Res. Tox.</i> (2004–2009), <i>Drug Metab. Dispos.</i> (2004–2009), and <i>Xenobiotica</i> (2004–2009).
Substrates	The focus was on drugs and other xenobiotics, excluding endogenous compounds except when these are used as drugs (e.g. estradiol).
Biological systems	The analysis was restricted to studies in humans and other mammals carried out either (i) <i>in vivo</i> , or (ii) in cellular systems, or (iii) at a subcellular or enzymatic level.
Sources of information	Metabolic trees or single enzyme studies.
Quality criteria	The papers had to report convincing biological and analytical conditions.

Rules for the analysis of papers and the classification of reactions

Rule 1	All reported metabolites were taken into account irrespective of their quantitative importance, provided their structural characterisation was unambiguous.
Rule 2	In each paper, the reported metabolites were classified in an ad hoc form according to the reaction type that produced them (Table 2). (Also see Rule 9)
Rule 3	The reported metabolites were also classified according to the enzyme (super)family or category that catalysed their formation (Table 2). (Also see Rule 8.)
Rule 4	The metabolites were also classified as being first generation, second generation, or third-plus generation ones.
Rule 5	When known or indicated as such, the metabolites were also marked as pharmacologically active or toxic and/or reactive.
Rule 6	Duplicate counts of substrates and metabolites were avoided. A substrate database served to identify substrates appearing in multiple papers. Also a given metabolite of a given substrate was counted only once when reported in several papers.
Rule 7	Regio- and stereoisomers were considered as distinct substrates (substrate selectivity) or metabolites (product selectivity).
Rule 8	When evidenced in the paper, or supported by existing knowledge and compatible with the biological conditions described in the paper, the formation of some metabolites was assigned to two enzyme (super)families or groups of enzymes. This was the case, for example, for the CYP- and FMO-catalysed oxygenation of some types of N- and S-containing functional groups, or the CYP- and peroxidases-catalysed formation of quinones from polyphenols.
Rule 9	The 2-electron reactions {–CHOH → >C=O}, {>C=O → –CHOH} and {–CHO → –COOH} were each counted as a single step. The same applied, for example, to the reactions {–NH → >NOH}, {–NHOH → –N=O}, {–NO ₂ → –N=O}, {–N=O → –NHOH} and {>NOH → >NH}.

to be included were selected according to the predefined criteria listed in Table 1, and no paper that fulfilled these criteria was discarded.

The metabolites reported in each paper were then taken into account and classified according to several criteria, namely:

- (i) the type of reaction that produced them (see below);
- (ii) the enzyme (super)family or category (see below) that produced them (see below);
- (iii) the metabolic generation to which they belonged;
- (iv) whether they were pharmacologically active;
- (v) whether they were toxic and/or chemically reactive (and hence potentially toxic).

Table 1 lists further rules whose implementation was necessary to proceed rationally and consistently.

Classification into reaction types

The first and major issue when setting up this meta-analysis was to define a list of metabolic reactions that was as comprehensive and realistic as possible without being exaggeratedly long. This list appears in Table 2 and is based on an extensive work (ca. 900 pages) co-authored by one of us (Testa) [38,39]. As is customary, metabolic reactions were classified into redox reactions, hydration and/or dehydration reactions, and conjugations. Carbon oxidations

being the most frequent ones were divided into Csp^3 - and Csp^2 -/ Csp -oxidations (Reaction types 01 and 02, respectively). Reductions at C5C moieties being rare and often catalysed by the gut flora, they were not classified separately but went to the 'other redox reactions' box (Reaction type 08). Redox reactions involving carbonyl compounds as products or substrates (i.e. oxidation of alcohols and aldehydes, reduction of aldehydes and ketones) were classified together (Reaction type 03). Two reaction types were dedicated to the oxidation and/or oxygenation of nitrogen atoms (Reaction types 04a and 05a), and two others to their reduction (Reaction types 04b and 05b); but note that each 2-electron step was considered as producing a distinct metabolite (Rule 9). The oxidation of diphenols, aromatic diamines and analogous compounds leading to quinones and analogues formed Reaction type 06a, whereas the reverse reaction was labelled as Reaction type 06b. Oxidations and reductions at sulphur atoms formed Reaction types 07a and 07b.

The classification of hydrolytic reactions needs little explanation. Note however that spontaneous reactions of hydration or dehydration were considered as a metabolic step and as such belonged into Reaction type 14. Conjugation reactions involving glutathione (Reaction type 24) deserve comments; here, the sequence from glutathione (GSH; reduced form) conjugation to the formation of mercapturic acids and even to thiols (owing to β -lyase catalysed C–S cleavage) was considered as a single pathway because it proved futile to do otherwise. Nonenzymatic or enzymatic conjugations with cysteine or N-acetylcysteine also belonged to Reaction type 24, as did GSH-mediated reactions of reduction. Reaction type 26a involved the formation of amino acid conjugates, while Reaction type 26b included other reactions subsequent to conjugation with coenzyme A, mainly β -oxidations but also, for example, 2-carbon elongation and chiral inversion of profens. Other conjugations belonged to Reaction type 28, mainly phosphorylations, coupling with endogenous carbonyls to form hydrazones, and carbon dioxide addition to primary and secondary amines to form carbamic acids (a straightforward non-enzymatic, reversible reaction). This Reaction type also included the few reactions of transamination seen in this analysis, because these reactions are catalysed by transferases.

Classification according to enzymes

The second objective was to classify the metabolites according to the enzyme category or categories that catalysed their formation, bearing in mind that nonenzymatic reactions (e.g. some hydrolyses, dehydrations and GSH conjugations, in addition to the formation of carbamic acids) were also reported and taken into account [40]. The enzyme classes we considered were the oxidoreductases (EC 1), the hydrolases (EC 3) and the transferases plus a few ligases (EC2 and EC 6) [38,39,41]. The oxidoreductases were the following.

CYPs; dehydrogenases and/or reductases, such as alcohol dehydrogenases (ADHs), aldehyde dehydrogenases (ALDHs), aldo-keto reductases (AKRs) and quinone reductases (NQOs); flavin-containing monooxygenases (FMOs); the molybdo-flavoenzymes [xanthine oxidoreductase (XOR) and aldehyde oxidoreductase (AOR)]; the peroxidases, including for example myeloperoxidase and prostaglandin G/H synthase but excluding CYPs when acting as

TABLE 2

Classification of reactions (columns 1 and 2) and their enzymes (column 3)

Redox reactions		
01	Oxidation of Csp^3	CYPs
02	Oxidation of Csp^2 and Csp	CYPs (AOR, XOR)
03	$-CHOH \leftrightarrow >C=O$ or $>C=O \rightarrow -COOH$	Dehydrogenases
04a	Oxidation of R_3N	CYPs, FMOs
04b	Reduction of N-oxides	Reductases
05a	Oxidation of $>NH$ or $>NOH$	CYPs (FMOs)
05b	Reduction of $-NO_2$ or $-N=O$ or $>NOH$	Reductases
06a	Oxidation to quinones or analogues	Peroxidases, CYPs
06b	Reduction of quinones or analogues	Reductases
07a	Oxidation of S atoms	CYPs, FMOs
07b	Reduction of S atoms	Reductases
08	Other redox reactions	Reductases, other OXRs
Hydrolytic reactions		
11	Hydrolysis of esters, lactones or inorganic esters	Hydrolases
12	Hydrolysis of amides, lactams or peptides	Hydrolases
13	Hydration of epoxides	Hydrolases
14	Other reactions of hydration, or reactions of dehydration	Hydrolases, non-enzymatic hydrolyses or (de)hydrations
Conjugation reactions		
21	O-Glucuronidation or glycosylations	UDP-Glucuronosyltransferases
22	Other glucuronidations (N, S or C)	UDP-Glucuronosyltransferases
23	Sulphonations	Sulphotransferases
24	Conjugations with glutathione and their sequels (including reductions)	GSH-transferases 1 associated enzymes
25	Acetylations	Acetyltransferases
26a	CoAs-Ligation 1 aminoacyl conjugations	Acyl-CoA ligases 1 associated enzymes
26b	CoAS-Ligation 1 other sequels	Acyl-CoA ligases 1 subsequent enzymes
27	Methylations	Methyltransferases
28	Other conjugations (PO_4 , $>C=O$, among others)	Other transferases or non-enzymatic reactions

peroxidases; other (often poorly defined) reductases; and other oxidoreductases, such as monoamine oxidases (MAOs).

Given the frequent absence of information in publications about the enzyme subclass or sub-subclass involved, we assigned all hydrolytic reactions to Class EC 3 (i.e. hydrolases), except for reactions demonstrated to be non-enzymatic. The classification of transferases was as follows: UDP-glucuronosyltransferases (UGTs); sulphotransferases (SULTs); glutathione S-transferases (GSTs) plus all enzymes involved in the sequence of reactions leading to the corresponding thiol (hydrolyses by peptidases, N-acetylation by cysteine S-conjugate NAT, and C-S cleavage by β -lyase); also, the enzymes involved in reduction reactions following conjugation with GSH; acetyltransferases, mainly N-acetyltransferases (NATs); acyl-coenzyme A ligases and the enzymes catalysing subsequent reactions (glycine N-acyltransferases and glutamine N-acyltransferases, which form the corresponding amino acid conjugates, and the various enzymes involved in β -oxidation or 2C-elongation or chiral inversion); methyltransferases (MTs), such as catechol-O-methyltransferase (COMT), the N-methyltransferases (NMTs), and the S-methyltransferases, such as thiol S-methyltransferase (TMT); and finally other transferases, such as phosphotransferases, together with non-enzymatic reactions; this category also included the non-enzymatic coupling of reactive substrates with glutathione, when investigated in abiotic media only.

In the vast majority of reactions, there was no ambiguity in assigning the formation of a given metabolite to a given enzyme

(super)family or category (e.g. to CYP, FMO or dehydrogenases and/or reductases), because either the enzyme (super)family was determined or no realistic alternative existed. However, a few cases (i.e. 200 metabolites, 3% of total metabolites) led to double assignment based on experimental evidence.

The relative significance of xenobiotic metabolic reactions

The example of [acetyl- $^2\text{H}_3$]phenacetin

To illustrate our approach, we begin with the encoding of a metabolomic investigation of [acetyl- $^2\text{H}_3$]phenacetin in humans [42]. The metabolites reported and listed in this article first had to be ordered into a metabolic tree where first-generation, second-generation and third plus-generation metabolites were logically and clearly arranged (Fig. 1). One regrets to note that a non-negligible proportion of metabolic trees appearing in the three source journals failed to satisfy this criterion and had to be rearranged in a sensible manner to enable encoding.

The metabolic reactions of [acetyl- $^2\text{H}_3$]phenacetin were then coded as shown. The original substrate underwent two first-generation reactions, namely amide hydrolysis (Reaction type 12), and oxidative O-desethylation (Reaction type 01), which yielded [acetyl- $^2\text{H}_3$]paracetamol (hence the code 'A' to signify activity). This metabolite underwent deacetylation followed by re-acetylation to (undeuterated) paracetamol (Reaction types 12 and 25). A toxicologically relevant reaction of paracetamol is its CYP-catalysed

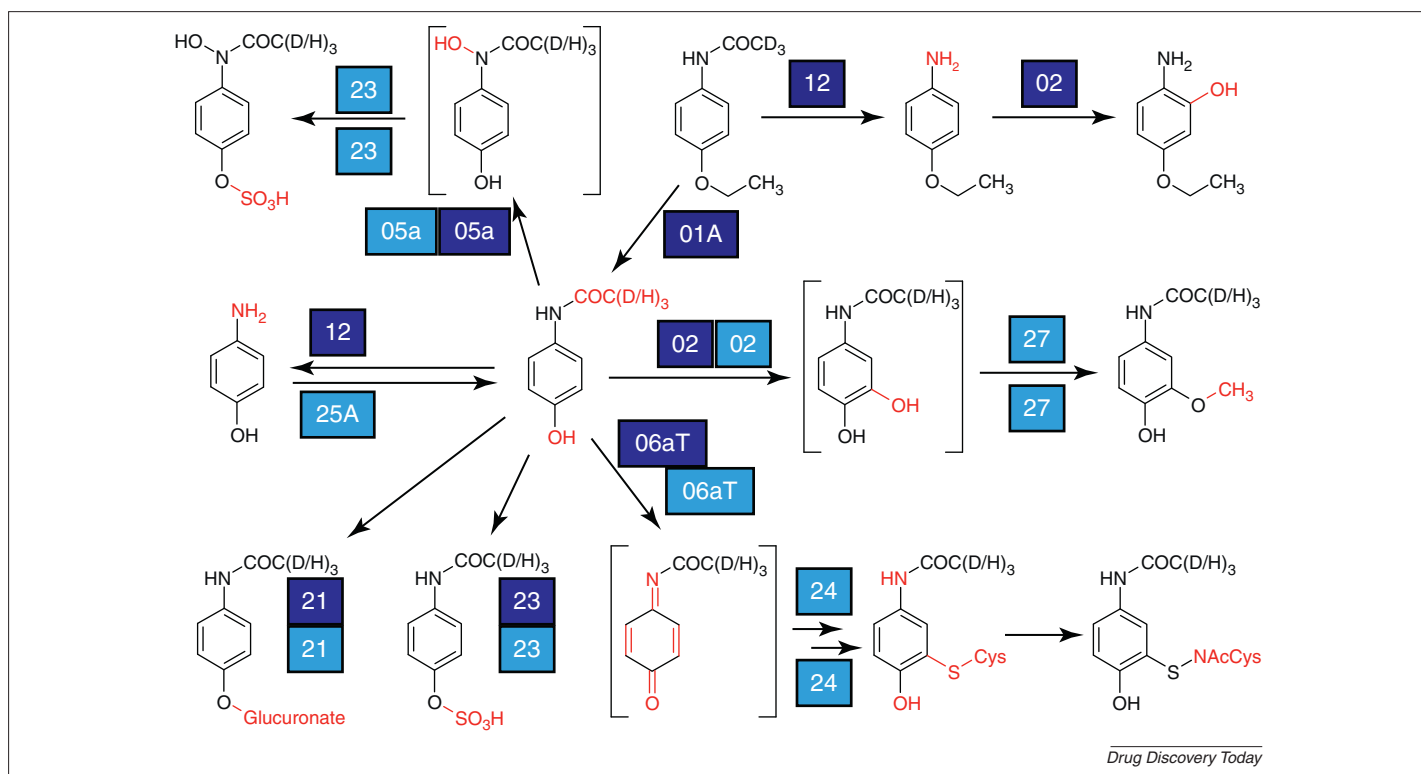


FIGURE 1

The metabolism of [acetyl- $^2\text{H}_3$]phenacetin [42] illustrates how metabolic reactions were classified. Dark blue, medium blue and light blue boxes correspond to the formation of first-generation, second-generation and third-plus-generation metabolites, respectively. The substrate is shown to undergo amide hydrolysis (Reaction 12) followed by aryl hydroxylation (Reaction 2). A competitive pathway is oxidative desethylation leading to the active [acetyl- $^2\text{H}_3$]paracetamol (Reaction 01A). The latter is the substrate of several reactions, for example hydrolytic desacetylation followed by N-acetylation to unlabelled paracetamol (Reactions 12 and 25A). Another reaction is oxidative toxification to the quinoneimine (Reactions 06aT) followed by glutathione conjugation (Reaction 24).

formation of a highly reactive and toxic quinoneimine (Reaction type 06a, plus the code 'T' for toxicity and/or reactivity). This reaction occurred twice, for the deuterated and undeuterated paracetamol. The individual reactions were then entered into the Microsoft SQL Database, while the global database report enabled immediate control of each entry.

A first quantitative overview

The first selection process produced a total of 903 papers (Supplementary material), of which 804 remained once redundant papers were discounted. A total of 1171 distinct substrates were found and analysed, 747 (~63%) being compounds of medicinal interest and 424 (~36%) other xenobiotics. Rather than being of negligible interest to medicinal chemists, these other xenobiotics were in fact bioactive ones, such as agrochemicals and pollutants whose toxification and detoxification mechanisms are of obvious relevance to drug discovery.

The 1171 substrates in the database yielded 6767 distinct metabolites (a mean of 5.78 per substrate); 201 (3%) of these were reported or known to be active, whereas 473 (7%) were reported to be toxic or featured a highly reactive functional group forming adducts (e.g. quinones).

'Phase I' versus 'Phase II' metabolic reactions: how real?

A global breakdown reveals the redox reactions accounted for 57% of all 6767 metabolites, hydrolyses for 10% and conjugations for 33%. However, such a picture is too coarse to impact on the 'Phase I versus Phase II' debate [43,44].

A more informative view can be found in Fig. 2, which shows how the relative importance of the three reaction classes evolves from the first to the second to later generations of metabolites (Table 3). First-generation metabolites were formed mainly (almost 70%) by redox reactions, whereas conjugates represented approximately 22%. In the second generation, the contribution of redox reactions had decreased to approximately 50% whereas conjugations had

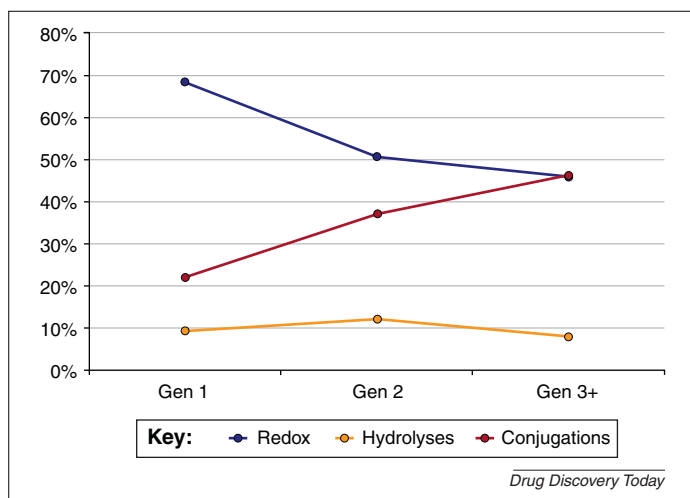


FIGURE 2

Distribution of metabolites according to generations. In each generation, the three percentages add up to 100% of the metabolites in that generation; this was done to avoid the artefactual bias caused by the smaller number of total metabolites in the second (2165) and subsequent (1792) generations compared with the first one (2810). Abbreviation: Gen: generation.

increased to 37%. In the third and later generations, both redox and conjugation reactions accounted for the same proportion (~46%) of metabolites. The proportion of metabolites generated by reactions of hydrolysis did not vary significantly from one generation to another and remained within a range of 8–12%.

Figure 2 demonstrates the inadequacy of the 'Phase I versus Phase II' classification, which assumes the biotransformation of xenobiotics to begin with redox or hydrolysis reactions ('Phase I'), followed in subsequent metabolic steps by conjugations ('Phase II'). Such a schematic view appears neat yet is misleading. Whereas Fig. 2 confirms that the contribution of 'Phase I' reactions decreases in later generations while that of conjugations increases, it also shows that the trend stops before the contribution of conjugations dominate that of redox reactions as implicit in the 'Phase I' and 'Phase II' terminology.

Distribution of metabolites according to reaction types and generations

Figure 3 breaks down the major reaction classes into the individual reaction types as listed in Table 2. The actual numbers of metabolites are reported in Table 3. Reactions of Csp³-, Csp²- and Csp-oxidation together accounted approximately 345% of all metabolites (Fig. 3), while redox reactions to and from the carbonyl group formed 8.3% of metabolites. Redox reactions at nitrogen-containing groups led to approximately 5.5% of metabolites, whereas redox reactions at sulphur-containing groups formed approximately 2.7% of metabolites. Redox reactions to form or reduce quinones and analogues accounted for 4.0% of all metabolites. Only 1.7% of metabolites were formed by unclassified redox reactions, mainly reduction of olefinic bonds.

Reactions of hydrolysis and hydration were mainly ester hydrolyses (~3.8%), while unclassified reactions such as oxime or imine hydrolysis, hydration of iminium groups or other electrophiles, hydrolytic dehalogenation or hydration of antitumor platinum compounds accounted for almost 2.8% of metabolites.

Conjugations reactions are dominated by glucuronidations (~14%), followed by enzymatic (or non-enzymatic) conjugations with GSH (or in a few cases with cysteine or N-acetyl-Cys) (8.0%). Sulphonations of hydroxy or amino groups represented 4.8% of metabolites.

Which reactions produced active metabolites?

Table 3 also features a breakdown of the pharmacologically active metabolites in our analysis. The total percentage (close to 3%) is in fact low, much lower than one would expect [8]. This appears to be a bias in the meta-analysis owing to several factors. Indeed, only approximately 64% of all substrates were compounds of medicinal interest, and many of these were developmental compounds the activity of whose metabolites was either unknown at the time or not reported for whatever reason. Nevertheless, a few conclusions can be proposed, keeping in mind the small number of metabolites included.

Table 3 reveals the vastly predominant role played by reactions of C-hydroxylation and ester hydrolyses in generating active metabolites (almost 36% and 37%, respectively). The role of ester hydrolysis should not come as a surprise given the current interest in prodrugs and specifically carrier-linker prodrugs [6,9,10]. As for the pharmacological significance of alkyl and aryl hydroxyla-

TABLE 3

Distribution of metabolites according to reaction types and generations

Reaction type	First generation	Second generation	Third-plus generation	Total (%)	Active metabolites	Toxic and/or reactive metabolites
01	^a 865	^a 354	^a 175	^a 1394 (20.6)	^a 59	^a 30
02	^a 617	^a 236	^a 89	^a 942 (13.9)	13	^a 71
03	^a 70	^a 231	^a 261	^a 562 (8.3)	7	12
04a	68	15	8	91 (1.35)	1	2
04b	8	1	0	9 (0.13)	1	0
05a	68	22	7	97 (1.43)	0	^a 39
05b	47	57	69	173 (2.56)	11	6
06a	52	92	84	228 (3.37)	1	^a 192
06b	10	9	23	42 (0.62)	5	0
07a	74	45	55	174 (2.57)	4	^a 45
07b	2	7	2	11 (0.16)	1	0
08	40	29	48	117 (1.73)	2	4
11	181	60	14	255 (3.77)	^a 74	7
12	54	28	36	118 (1.74)	5	1
13	1	80	29	110 (1.63)	0	2
14	28	95	65	188 (2.78)	4	11
21	^a 250	^a 334	^a 277	^a 861 (12.7)	2	7
22	56	24	12	92 (1.36)	0	0
23	82	141	103	326 (4.82)	0	18
24	^a 94	^a 195	^a 254	^a 543 (8.02)	0	13
25	36	28	38	102 (1.51)	3	7
26a	24	12	38	74 (1.09)	0	0
26b	28	4	16	48 (0.71)	1	3
27	31	56	62	149 (2.20)	3	2
28	24	10	27	61 (0.90)	4	1
Totals (%)	2810 (41.5)	2165 (32.0)	1792 (26.5)	6767 (100)	201 (2.97)	473 (6.99)

^a Reactions playing a significant role.

tions, this was owing to the hydroxylated metabolite(s) retaining the target affinity and pharmacological activity of the parent compound.

The low proportion of pharmacologically active conjugates does not come as a surprise, as only a few examples are known (e.g. morphine 6-O-glucuronide). This is also in line with the fact that most of the 201 active metabolites counted here (143 = 71%) were produced as first-generation metabolites. Only 42 (21%) and 16 (8%) were formed in the second generation or later, respectively.

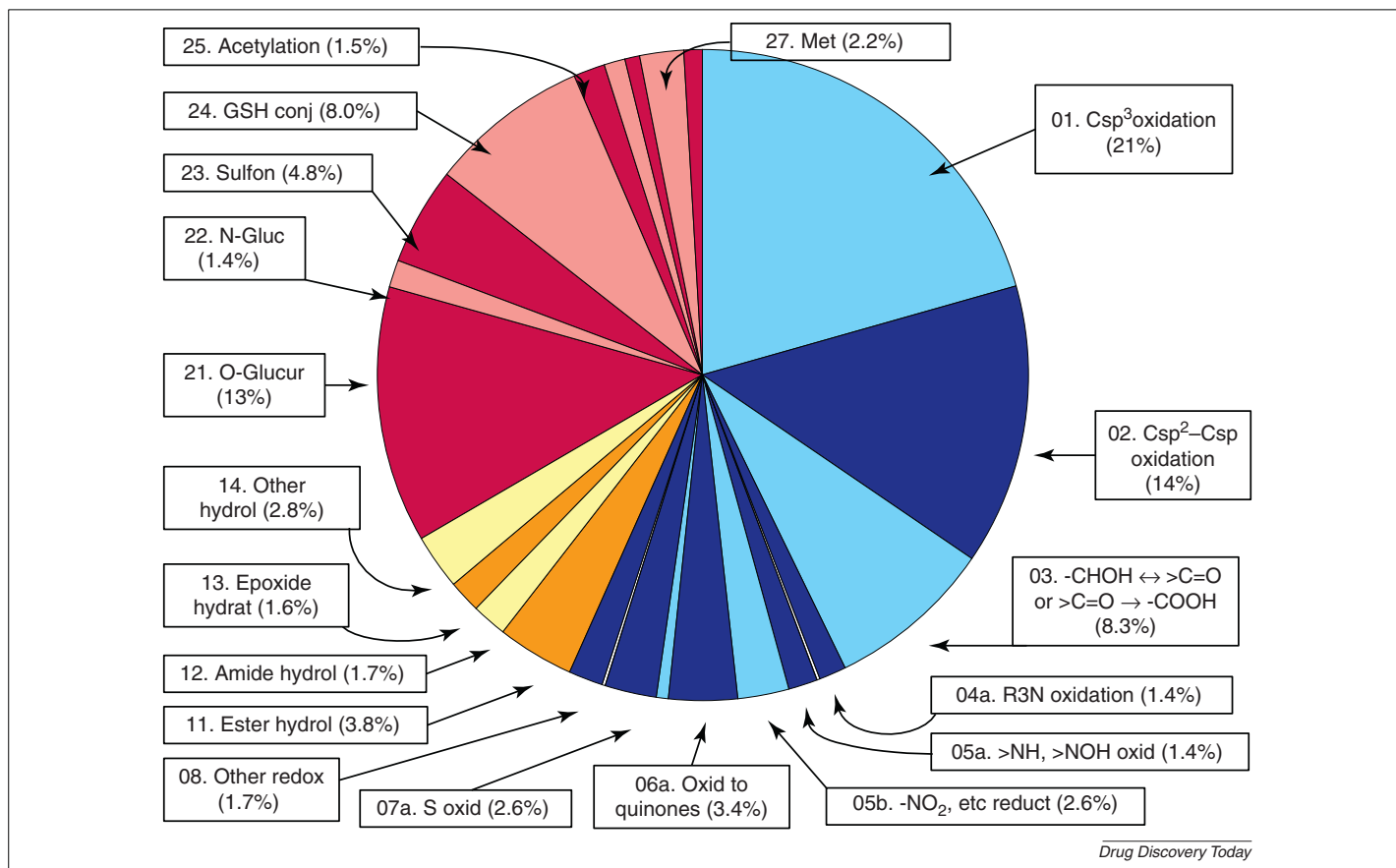
Which reactions produced toxic and/or chemically reactive (and hence potentially toxic)?

Table 3 also reports the detection and detailed breakdown of 473 toxic or reactive metabolites, accounting for approximately 7% of the total number of metabolites. To be counted in this class, metabolites had to either be reported or known to be toxic or reactive. In many cases, chemical reactivity (mostly strong electrophilicity) was demonstrated by the formation of adducts with macromolecules or nucleophiles, such as thiols.

Csp²- and Csp-oxidations accounted for 15% of reactive metabolites (Fig. 4), whereas N- and S-oxidations together yielded a

total percentage of almost 18%. However, the most remarkable result is the fact that quinones and analogues accounted for over 40% of all reactive metabolites. This figure should send a strong warning to all medicinal chemists, that the possible para- or ortho-hydroxylation of phenols or arylamines is just a single 2-electron step removed from an strong adduct-forming electrophile. Such a reaction of toxification is often catalysed by CYPs, but the significance of peroxidases, such as prostaglandin G/H synthase and myeloperoxidase (which is responsible for the final reaction of toxification of benzene to benzoquinone) is often neglected. This is all the more worrying because the tissue distribution of peroxidases differs markedly from that of CYPs, not to mention that these enzymes are far from receiving the attention they deserve.

The reactive metabolites formed by Reaction 02 were epoxides stable enough to be detected, namely those formed from polycyclic aromatic hydrocarbons (PAHs) and mainly their dihydrodiol-epoxides. The quantitative significance of such ultimate carcinogens in our analysis is explained by the large number of papers focused on the toxification of environmentally relevant PAHs. The oxygenation of primary amines to hydroxylamines and possibly nitroso compounds (Reaction 05a), or of thiols to sul-

**FIGURE 3**

Distribution of metabolites according to reaction types. The percentages shown refer to 6767 metabolites = 100%; they were rounded to two significant digits. The colour code is as follows: Redox reactions blue; hydrolyses yellow; conjugations red. Alternating dark and light fields are used simply for graphical clarity. Starting in the upper-right quadrant, the boxes show (i) Reactions 01 (*sp*³-carbon oxidation), (ii) Reactions 02 (*sp*²- and *sp*-carbon oxidation), (iii) Reactions 03 (reversible alcohol (de)hydrogenation and aldehyde dehydrogenation), (iv) Reactions 04a (oxygenation of tertiary amines), (v) Reactions 04b (reduction of N-oxides, no box shown), (vi) Reactions 05a (oxidation of >NH and >NOH groups), (vii) Reactions 05b (reduction of -NO₂ or -N=O or >NOH groups), (viii) Reactions 06a (oxidation to quinones or analogues), (ix) Reactions 06b (reduction of quinones or analogues, no box shown), (x) Reactions 07a (oxidation of sulphur atoms), (xi) Reactions 07b (reduction of sulphur atoms, no box shown), (xii) Reactions 08 (other redox reactions), (m) Reactions 11 (hydrolysis of esters, lactones or inorganic esters), (xiii) Reactions 12 (hydrolysis of amides, lactams or peptides), (xiv) Reactions 13 (hydration of epoxides), (xv) Reactions 14 (other reactions of hydration, or reactions of dehydration), (xvi) Reactions 21 (O-glucuronidation or glycosylations), (xvii) Reactions 22 (glucuronidation of N, S or C atoms), (xviii) Reactions 23 (sulphonations), (xix) Reactions 24 (Conjugations with glutathione and their sequels), (xx) Reactions 25 (acetylations), (xxi) Reactions 26a (coenzyme A-ligation 1 aminoacyl conjugations, no box shown), (xxii) Reactions 26b (coenzyme A-ligation 1 other sequels, no box shown), (xxiii) Reactions 27 (methylations), and (xxiv) Reactions 28 (other conjugations (e.g. phosphorylation, CO₂ addition, carbonyl conjugation, no box shown)). *Abbreviation:* GSH: glutathione.

phenic acids (Reaction 07a) each account for almost 10% of reactive metabolites identified in this analysis.

Conjugation reactions play a comparatively modest role in toxification reactions because they account for only 11% of the toxic or reactive metabolites seen in this analysis. A limited number of reactive metabolites are known to be produced by sulphonation of an alcohol group (Reaction 23), the resulting sulphate ester being an electrophilic alkylating agent. Some acyl-glucuronides are known to be reactive (Reaction 21), as are a few GSH conjugates (Reaction 24).

At which stage were these toxic and/or reactive metabolites produced?

Medicinal chemists and other scientists in drug discovery need to know about the formation and structure of reactive metabolites, as discussed later. This however might not be sufficient as improperly

designed investigations might fail to reveal their formation. A key factor in this context is the metabolic generation at which a reactive metabolite has the greatest probability of formation. Among the 473 toxic and/or chemically reactive (and hence potentially toxic) metabolites counted in this meta-analysis, 202 (43%) were formed as first-generation metabolites, 161 (34%) as second-generation metabolites, and 110 (23%) as third- or later-generation metabolites. Interestingly, this distribution is practically identical with that of the total 6767 metabolites counted in the meta-analysis (Table 3). In other words, reactive metabolites have practically the same likelihood to be formed at any generation in a metabolic tree. This is clearly relevant when using metabolism-predicting software, or for studies in subcellular biosystems carried out with a single enzyme type (e.g. CYPs) or for a too short duration or without trapping agents. Both approaches indeed might well fail to detect reactive metabolites and to issue a proper warning, as discussed below.

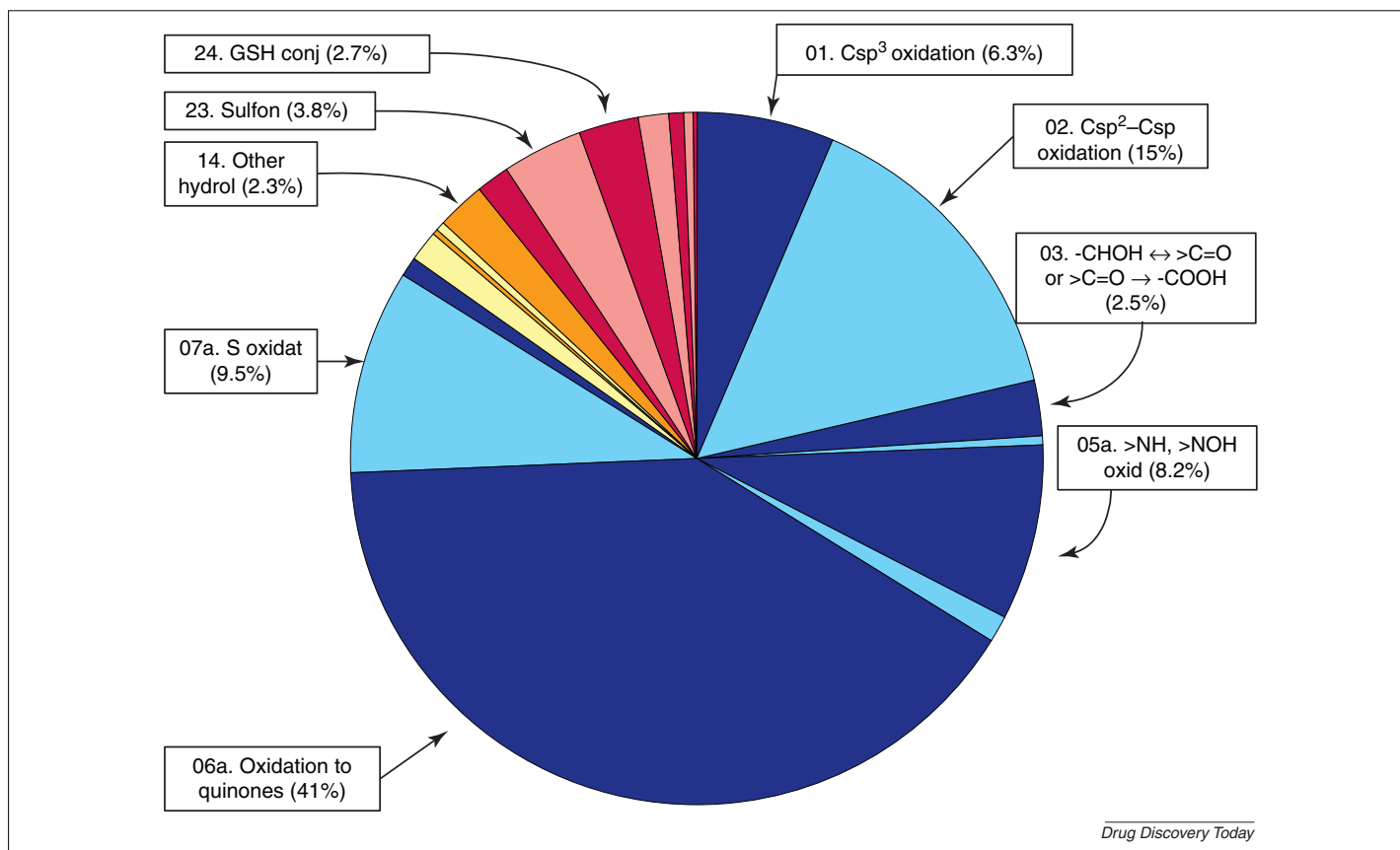


FIGURE 4

Which reactions formed reactive and/or toxic metabolites? (Reminder: 7% of all metabolites). The percentages shown refer to 473 metabolites = 100%; they were rounded to two significant digits. The colour code is as follows: Redox reactions blue; hydrolyses yellow; conjugations red. Alternating dark and light fields are used simply for graphical clarity. Starting in the upper-right quadrant, the boxes show (i) Reactions 01 (*sp*³-carbon oxidation), (ii) Reactions 02 (*sp*²- and *sp*-carbon oxidation), (iii) Reactions 03 (reversible alcohol (de)hydrogenation and aldehyde dehydrogenation), (iv) Reactions 04a (oxygenation of tertiary amines, no box shown), (v) Reactions 05a (oxidation of >NH and >NOH groups), (vi) Reactions 05b (reduction of -NO₂ or -N=O or >NOH groups, no box shown), (vii) Reactions 06a (oxidation to quinones or analogues), (viii) Reactions 07a (oxidation of sulphur atoms), (ix) Reactions 08 (other redox reactions, no box shown), (x) Reactions 11 (hydrolysis of esters, lactones or inorganic esters, no box shown), (xi) Reactions 12 (hydrolysis of amides, lactams or peptides, no box shown), (xii) Reactions 13 (hydration of epoxides, no box shown), (xiii) Reactions 14 (other reactions of hydration, or reactions of dehydration), (xiv) Reactions 21 (O-glucuronidation or glycosylations, no box shown), (xv) Reactions 23 (sulphonations), (xvi) Reactions 24 (Conjugations with glutathione and their sequels), (xvii) Reactions 25 (acetylations, no box shown), (xviii) Reactions 26b (coenzyme A-ligation 1 other sequels, no box shown), (xix) Reactions 27 (methylations, no box shown), and (xx) Reactions 28 (other conjugations (e.g. phosphorylation, CO₂ addition, carbonyl conjugation, no box shown)). *Abbreviation:* GSH: glutathione.

Distribution of metabolites according to enzyme (super) families or categories

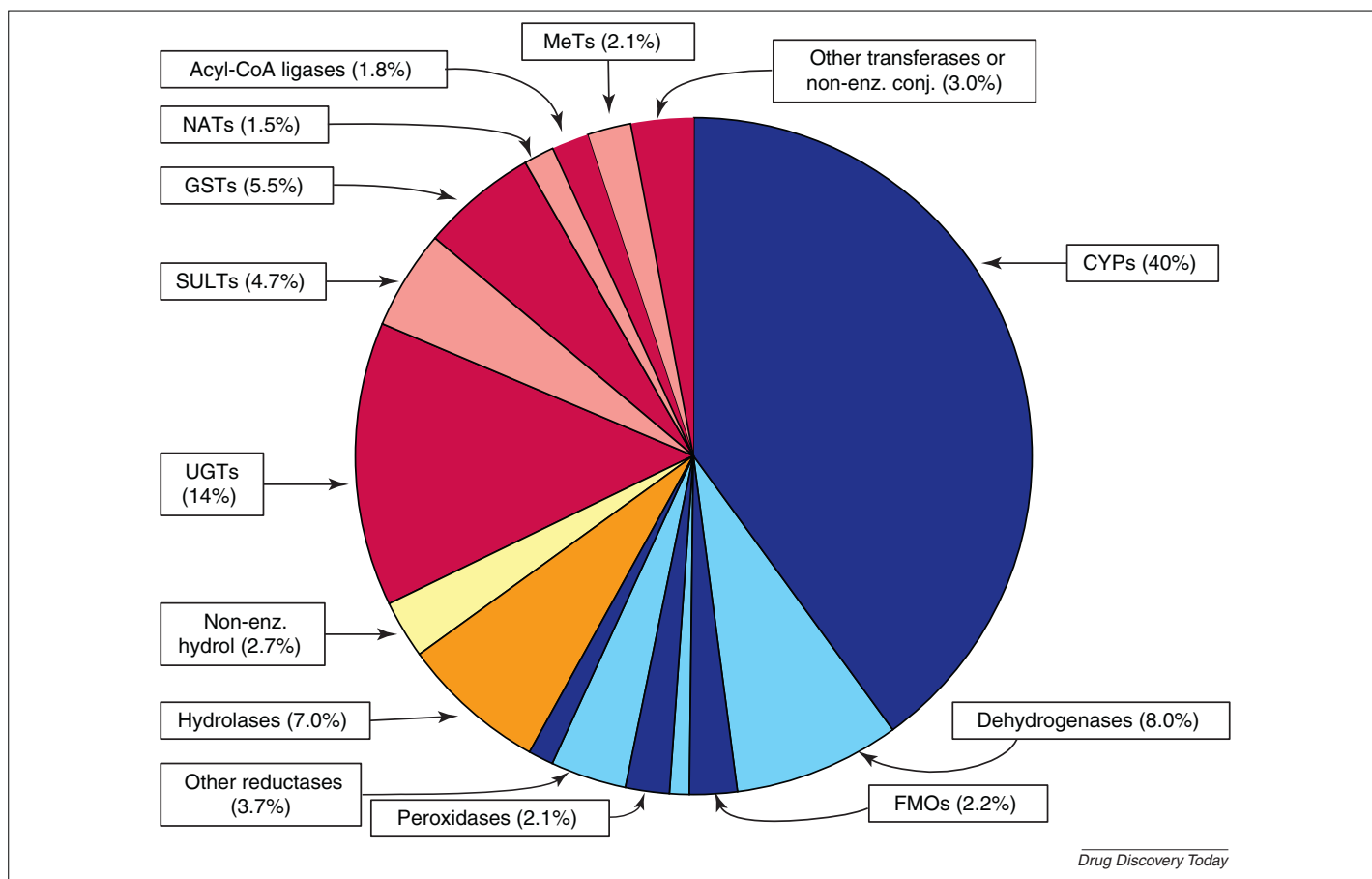
The above discussion was centred on metabolic reactions, but what about the enzymes that catalyse them? As explained, there was an almost one-to-one relation in our approach between metabolic reactions and enzymes, since in only 200 cases (3%) was a metabolite produced by enzymes classified into two distinct categories. In most cases, experimental proof was indeed provided for the involvement of two distinct enzyme systems, (e.g. CYPs and FMOs, or CYPs and peroxidases). In other cases, the assay conditions were clearly suggestive of a dual enzymatic catalysis.

By far the most significant enzymes clearly appear to be the CYPs, because they accounted for 40% of all 6767 metabolites and 6967 enzyme occurrences (Fig. 5). This figure is well below the proportion of drugs (~80%) known to be CYP substrate [45,46], but as stated above our meta-analysis counted metabolites and not substrates. Second in importance were the UGTs (almost 14%), followed by the dehydrogenases and hydrolases (~8%

and 7%, respectively). Noteworthy are also the GSTs and the SULTs (~5.5% and 4.7%, respectively). The other enzymes played modest roles, in particular the molybdo-flavoenzymes and the other OXRs, whose label does not appear on Fig. 5 and which each accounted for 1%.

Concluding remarks: implications for drug discovery

A word of caution is needed before discussing some implications of this work. Indeed, no analysis or meta-analysis can be better than the data it is based on. This is a problem also faced by and discussed in other reviews, for example two important ones on inhibition-based drug-drug interactions [47,48]. Whether any database assembled for a meta-analysis suffers from a bias created by the existence of unpublished data is a reality. The best that can be done, and was done here, to minimise biases is to assemble a database from the primary literature published in major, peer-reviewed journals, the selection of papers being carried out according to rigorous, previously defined criteria.

**FIGURE 5**

Distribution of metabolites according to enzyme (super) families or categories. The percentages shown refer to 6967 enzyme occurrences = 100%; they were rounded to two significant digits. The colour code is as follows: Redox reactions blue; hydrolyses yellow; conjugations red. Alternating dark and light fields are used simply for graphical clarity. Starting in the upper-right quadrant, the boxes show (i) Reactions catalysed by cytochromes P450, (ii) Reactions catalysed by dehydrogenases, (iii) Reactions catalysed by flavin-containing monooxygenases, (iv) Reactions catalysed by xanthine oxidoreductase and aldehyde oxidase (no box shown), (v) Reactions catalysed by peroxidases, (vi) Reactions catalysed by other reductases, (vii) Reactions catalysed by other oxidoreductases or reactions of auto-oxidation (no box shown), (viii) Reactions catalysed by hydrolases, (ix) Non-enzymatic hydrolyses or (de)hydrations, (x) Reactions catalysed by UDP-glucuronosyltransferases, (xi) Reactions catalysed by sulphotransferases, (xii) Reactions catalysed by glutathione S-transferases and associated enzymes, (xiii) Reactions catalysed by N-acetyltransferases, (xiv) Reactions catalysed by acyl-coenzyme A ligases 1 associated enzymes, (xv) Reactions catalysed by methyltransferases, (xvi) Reactions catalysed by other transferases or non-enzymatic reactions of conjugations. *Abbreviations:* CYP, cytochrome P450; FMO, flavin-containing monooxygenases; GST, glutathione S-transferase; NATs, N-acetyltransferases; SULTs, sulphotransferases; UGTs, UDP-glucuronosyltransferases.

The evolutionary logic and inevitability of xenobiotic metabolism

Given that the endless accumulation of even nontoxic xenobiotics in organisms is incompatible with their survival, natural selection has led to the evolution of protective strategies of which metabolism is but one [49]. Indeed, we owe our current biological protection against foreign compounds to the innumerable natural xenobiotics in existence before the appearance of humankind [50]. Schematically, three protective strategies have emerged: (i) passively or actively inhibited entry into an organism or organ, (ii) passive or active excretion (physical elimination), and (iii) metabolism (chemical elimination) as synonymous with biotransformation. In a global perspective, the biotransformation strategy has evolved to increase the hydrophilicity of lipophilic xenobiotics and hence facilitate their excretion by the renal, biliary or other routes [51–53].

However, this view is simplistic and outdated, and several biological mechanisms intervene in metabolite disposition, such that many metabolites do indeed circulate rather than being

rapidly excreted [54]. This situation is in fact a favourable one as far as drug discovery is concerned. However, it also implies that practically all new chemical entities, lead compounds, clinical candidates, drug candidates and drugs are susceptible to biotransformation. However, few exceptions to this rule exist, namely highly polar drugs, such as zanamivir.

The relative importance of drug-metabolising enzymes in drug discovery

Numerous enzyme superfamilies and families have a role in drug metabolism, but the relative involvement of these enzymes, both in quantitative and qualitative terms, remains a matter of debate. When listening to some medicinal chemists, one gets the feeling that drug metabolism begins and ends with cytochrome P450, and that the word ‘metabolism’ implicitly implies ‘by cytochrome P450’.

The present meta-analysis does indeed confirm the primary role of CYP-catalysed reactions in *in vitro* and *in vivo* systems, but it also

demonstrates that the role of non-CYP enzymes (i.e. other oxidoreductases, esterases and transferases), adds up to a significant proportion of all drug metabolism reactions. Our analysis is particularly eloquent in this regard, showing that almost 58% of first-generation metabolites are indeed produced by CYPs, but that the contribution of this superfamily strongly decreases in the second (~33%) and mainly third generations (~21%). This relative decrease is compensated by an increased involvement of transferases mainly, but also of non-CYP oxidoreductases. The latter groups of enzymes seem to attract far less attention than CYPs, and much remains to be discovered in their enzymology and involvement in drug discovery.

Two main implications emerge from these results, (i) to use biological and computational tools better able to reveal potentially toxic metabolites, and (ii) an urgent need to offer (future) medicinal chemists a proper education in drug metabolism and multiplicity of enzymes, their consequences and the factors affecting them.

Remember the poet: 'Not known, because not looked for' [55].

Some comments about toxic and/or chemically reactive (and hence potentially toxic) metabolites

As illustrated quantitatively above, metabolic detoxification could fail in several cases when reactive or more lipophilic metabolites are produced [56]. The latter case is sometimes seen when a drug enters pathways of lipid biochemistry following transient conjugation with Coenzyme A [57], but such occurrences are rare as evidenced here (Table 3, Reaction 026b).

A markedly greater source of worry and potential toxicity is seen with redox reactions, most significantly with the formation of quinones, quinonimines, quinonimides and quinone-diimines, which accounted for 40% of all toxic and/or reactive metabolites identified in this work. As discussed above, the ortho- or para-hydroxylation of medicinal phenols or arylamines will yield metabolites just a single 2-electron step removed from such reactive electrophiles. The demonstrated or strongly suspected involvement of peroxidases in their formation, together with a cellular and tissular distribution differing from that of CYPs, is in line with the increased role of non-CYP oxidoreductases in the formation of second- and later-generation metabolites. One of our main conclusions is therefore that medicinal (bio)chemists should be more attentive to the formation of chemically reactive metabolites. These indeed seem to account for an unknown (but certainly not negligible) proportion of rare yet dangerous toxic effects seen during clinical trials and even post-marketing.

How can the in silico prediction of metabolism issue realistic warnings?

There is a strong incentive in drug discovery to anticipate the biotransformation of any given compound, including the generation of reactive metabolites [32]. Human expertise certainly remains irreplaceable, but its efficiency can be greatly improved by *in silico* methods aimed at providing reliable and versatile metabolic predictions. What must be realised, however, is that such predictions are not necessarily infallible and remain essentially qualitative.

In a schematic manner, *in silico* methods can be classified into specific ('local') and comprehensive ('global') [35,58]. Specific

predictive methods are applicable to simple biological systems, such as a single enzyme or a single reaction; they include quantitative structure-metabolism relationships, 3-dimensional quantitative structure-metabolism relationships (3D-QSMR), quantum mechanical calculations, molecular modelling of enzymes and ligand docking, and combinations thereof. Some of these methods will predict whether a given compound is a substrate of a given enzyme or reaction, other make predictions about the regioselectivity of a given reaction. The fact remains, however, that most specific systems (e.g. MetaSite™) focus on cytochromes P450 (CYPs) [59–62], with some promising molecular modelling studies involving, [e.g. UDP-glucuronosyltransferases (UGTs), glutathione S-transferases (GSTs) and carboxylesterases (CEs)]. The marked prominence of CYP-catalysed reactions in predictive algorithms is well understandable given the comparatively large number of xenobiotic-metabolising CYPs in mammals and the large proportion of drugs (~80%) known to be substrates thereof [45,46]. Nevertheless, such methods have a distinct probability of failing to predict the formation of reactive electrophilic metabolites, especially when formed as second- or later-generation metabolites.

Global methods are applicable to versatile biological systems (many enzymes, many reactions, etc.) and to series of compounds with broad chemical diversity [63]. They include rule-based expert systems and their knowledge and databases, and their aim is to predict both Phase I and Phase II metabolites. Provided their knowledge databases and rules are comprehensive enough, such global systems offer a fair chance of identifying toxic metabolites provided their use includes second- and later generation metabolites.

How can the in vitro studies of metabolism issue adequate warnings?

The outcome of metabolism studies carried out with subcellular systems depend heavily on the nature (9000 g supernatant (S9), microsomes, cytosol or expressed enzymes) and origin of the systems, the cofactors added, and the duration of incubations, to name some factors. Perforce, such biological media cannot offer the complete enzymatic spectrum faced by substrates *in vivo* and are bound to generate only a fraction of the total number of metabolites produced *in vivo*. Thus, several second- and later-generation metabolites will not be formed during short incubations, in the absence of suitable cofactors, or if the biosystem does not contain the relevant enzyme(s). For example, electrophiles generated by peroxidases will not be formed in the traditional metabolic investigations using human or animal hepatic microsomes. Among the papers analysed, more than half used hepatic S9 or microsomes, meaning that their objective was not to gain a comprehensive view of the metabolism. One should not wonder, therefore, that the formation of toxic metabolites from some developmental compounds is detected only during advanced *in vivo* animal studies or even later.

It seems to us that the use of pluripotent *in vitro* biosystems would help in the early identification of reactive metabolites. As a matter of fact, it was a surprise to find that so few studies in our analysis (12.7%) used human or animal hepatocytes. Primary hepatocyte cultures might be more expensive to acquire or technically more demanding to prepare than subcellular homogenates, but their battery of enzymes is richer and they enable for

longer durations of incubation. Increasing, diversifying and optimising their use should become an objective in drug discovery.

Educating medicinal chemists and other drug researchers

Drug discovery begins with human scientists in their various disciplines and functions. What is more, these scientists must be prepared for their job and vocation, meaning that they must assimilate 'text and context' in preparation of, and during, their tenure as drug researchers. The education of undergraduates, graduates and professionals is a crucial factor in the future success of drug discovery projects. As this meta-analysis confirms, new chemical entities, lead compounds and other candidates face a huge variety of enzymes and metabolic reactions, sometimes with unwanted consequences if not attrition. When and how to obtain a realistic view of the metabolic fate of potential drugs has been considered above, but we add here that a condition of success of any drug discovery programme is the involvement of medicinal chemists having acquired a broad understanding of the discipline of absorption, distribution, metabolism, excretion and toxicity (ADMET), and having been offered a first opportunity to do so during their studies.

This leads us to wonder about the adequacy of the biochemical and biomedical exposure offered (if at all) to chemistry students and practising medicinal chemists. In our experience, this factor

remains a seriously underrated component in the academic and continuous education of medicinal chemists. It is hoped that the awareness of research directors will continue to grow.

To close, the present meta-analysis and its implications should help drug discovery scientists (drug designers, medicinal chemists, biochemists, biologists, among others) gain a broader and more balanced view of the numerous xenobiotic-metabolising reactions and enzymes they might encounter. *In silico* and pluripotent experimental methodologies together with more ambitious educational programmes should provide a synergy towards more selective ADMET screens in drug discovery.

Supplementary material

The full references to the 903 papers analysed in this work can be found at http://www.ddl.unimi.it/Ref_Database.pdf.

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