ELSEVIER

Role of pharmacologically active metabolites in drug discovery and development

Aberra Fura

Department of Metabolism and Pharmacokinetics, Pharmaceutical Research Institute, Bristol Myers Squibb, P. O. Box 5400, Princeton, NJ 08534, USA

Pharmacologically active metabolites can contribute significantly to the overall therapeutic and adverse effects of drugs. Therefore, to fully understand the mechanism of action of drugs, it is important to recognize the role of active metabolites. Active metabolites can also be developed as drugs in their own right. Using illustrative examples, this paper discusses a variety of biotransformation reactions that produce active metabolites and their structure–activity relationships. The paper also describes the role and significance of active metabolites in drug discovery and development, various experimental observations that can be used as indicators of their presence, and methods that can be used to assess their biological activities and contribution to the overall therapeutic and adverse effects of drugs.

Xenobiotic substances such as drugs undergo an array of biotransformation reactions. The body uses these reactions as a major line of defense against any invading xenobiotic substances, including drugs and environmental chemicals. To catalyze these reactions, the body is armed with a battery of enzyme systems. For the most part, the biotransformation products are more water soluble than the parent molecule and can thus be handled more easily by excretory organs such as the kidney. Total xenobiotic load is also minimized by the transformation of a xenobiotic substance to multiple products. Consequently, metabolism is the major clearance mechanism for most drugs [1–3].

Biotransformation reactions can be accompanied by various events, such as the formation of chemically stable metabolites, which are devoid of pharmacological or toxicological activities, or the generation of short-lived chemically-reactive metabolites, which can lead to toxicological activation [4–7]. Biotransformation reactions can also result in the formation of chemically stable metabolites with pharmacological activity (on or off target); this is the main focus of this review [1,4,5,8,9].

Pharmacologically active metabolite can be significantly or entirely responsible for the therapeutic effect of a drug (on-target activity) or they could have off-target activities unrelated to the action of the parent molecule and in some instances they might even reverse the pharmacological action of the parent drug. In fact, it is estimated that ~22% of the top 50 drugs prescribed in the USA in 2003 undergo biotransformation into metabolites that play significant roles in the pharmacological actions of the corresponding drugs or generate pharmacologically active metabolites that are developed as drugs in their own right. Important examples include, atorvastatin (Liptor®) [10,11], simavastatin (Zocor®) [11,12], fluoxetine (Prozac®) [13], fexofenadine (Allegra®) [14,15] and cetirizine (Zyrtec®) [14].

Because of the impact of biotransformation reactions on the fate of drugs as a whole (e.g. drug clearance and bioavailability), *in vitro* (e.g. metabolic stability screening) and *in vivo* metabolism and pharmacokinetic studies are conducted as part of the drug discovery and development processes [2,3,16]. The main objective of these studies, particularly at the drug discovery stage, is to monitor the dynamics of the parent compounds in a relevant *in vitro* and *in vivo* system in a timeline that is consistent with the fast pace of the drug discovery process. As a result, the necessary information regarding the identity and characteristics of metabolites, which might put the efficacy and safety profiles of drug candidates in perspective, is usually not available during these standard pharmacokinetic and metabolic stability screening. More often than not, such screens overlook, for example, the significant role that pharmacologically active metabolites might play in the efficacy of the parent molecule or as independent entities. However, to improve the decision-making process at the drug discovery stage and to design appropriate clinical studies, it is important to recognize the

Corresponding author: Fura, A. (aberra.fura@bms.com).

1359-6446/06/$ – see front matter PII: S1359-6446(05)03681-0

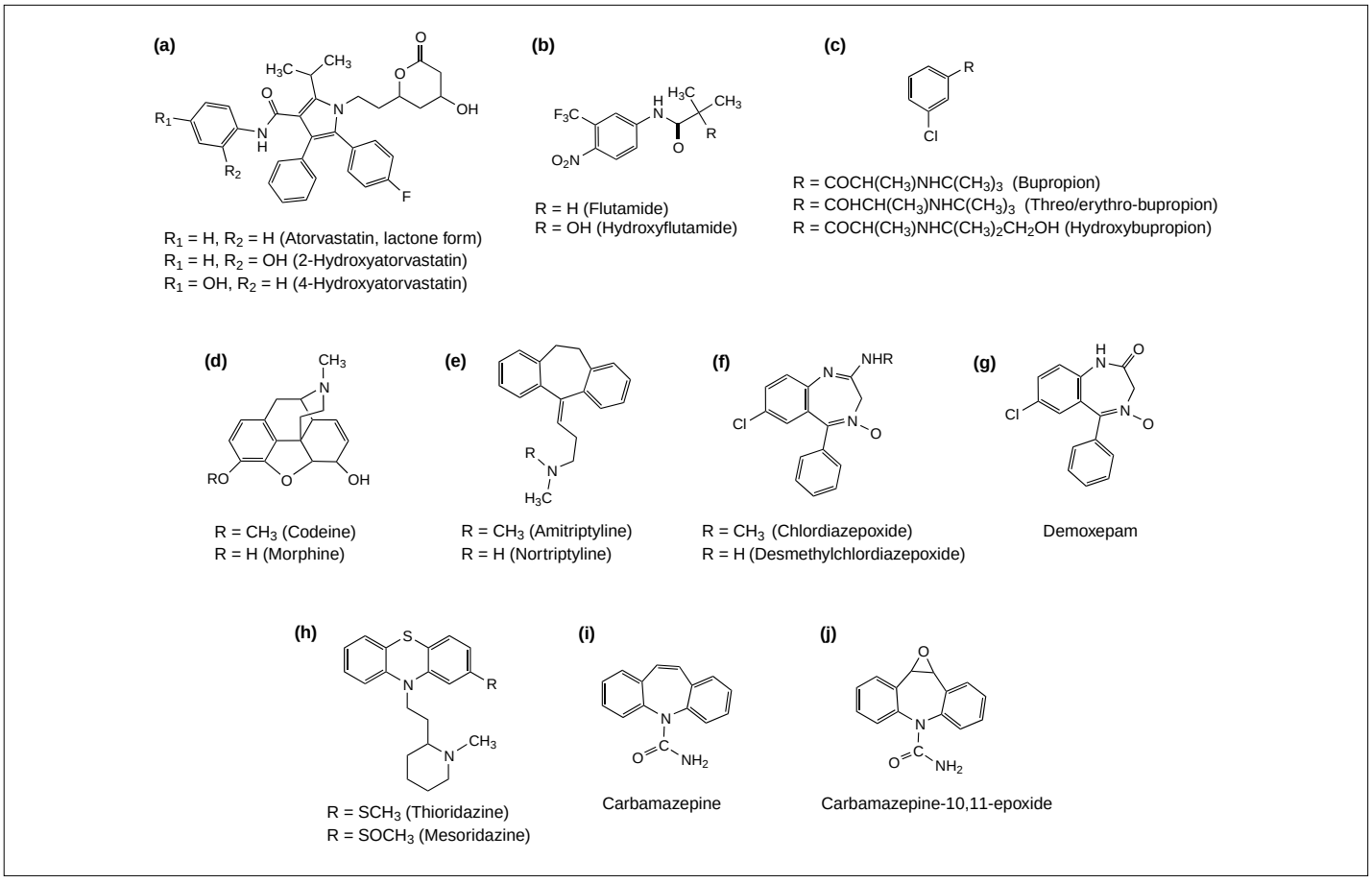

FIGURE 1

Examples of metabolites obtained through 'phase I' biotransformation reactions leading to pharmacological activation.

role of pharmacologically active metabolites early on, and put in place appropriate assays to detect and evaluate them [1,4,5,8]. To that end, this review will describe the role and significance of active metabolites in drug discovery and development processes. Examples of biotransformation reactions that result in the formation of active metabolites and their detection will also be discussed.

Biotransformation reactions leading to the formation of active metabolites

In general, biotransformation reactions are divided into two broad categories known as 'phase I' and 'phase II'. Phase I reactions are functionalization reactions that introduce polar chemical moieties either by inserting new polar functional groups or by interchanging or unmasking existing functional groups via oxidation, reduction and hydrolytic reactions. Phase I reactions are mediated by enzymes such as cytochrome P450 (CYP), flavin-containing monooxygenase (FMO), esterases and amidases. CYP enzymes are by far the most important enzymes responsible for the pharmacological activation of many drugs [1,4,5,8]. In phase II reactions, small endogenous polar molecules (e.g. glucuronic acid and sulfate) conjugate with the functional groups formed during phase I reactions. Direct conjugation of the endogenous molecules can also occur if the compound already contains appropriate functional groups. These conjugative reactions are mediated by enzymes such as glucuronosyltransferase, sulfotransferase and *N*-acetyltransferase.

In a traditional prodrug-based approach to drug discovery, biotransformation reactions are used to convert pharmacologically inactive compounds to pharmacologically active metabolites. These reactions are usually mediated by a broad class of hydrolytic enzymes, such as esterases, amidases and phosphotases, although the conversion of a prodrug to the corresponding active drug can also occur nonenzymatically. The main focus of the subsequent discussion is the formation of pharmacologically active metabolites from pharmacologically active compounds through phase I (oxidative or reductive) and phase II (conjugative) metabolism.

Biotransformation reactions leading to pharmacological activation of drugs can involve aliphatic or aromatic carbon hydroxylation, *N*-, *O*- or *S*-dealkylation, epoxidation, heteroatom oxidation (N, S, and P), reduction, glucuronidation, sulfation and acetylation, and so on. For example, atorvastatin, a cholesterol-lowering drug, is metabolized to two active metabolites, 2- and 4-hydroxyatorvastatin via aromatic hydroxylation (Figure 1a) [10,11]. Flutamide, a drug used for the treatment of prostate cancer, undergoes hydroxylation at the alkyl side chain to hydroxyflutamide, a metabolite that is more active and has longer duration of action compared with the parent drug (Figure 1b) [17]. Aliphatic hydroxylation of bupropion (an antidepressant) generates an active metabolite, hydroxybupropion (Figure 1c) [18]. Bupropion also forms two other active metabolites, namely threohydrobupropion and erythrohydrobupropion via carbonyl reduction (Figure 1c) [18]. *O*-demethylation of codeine (an analgesic) gives

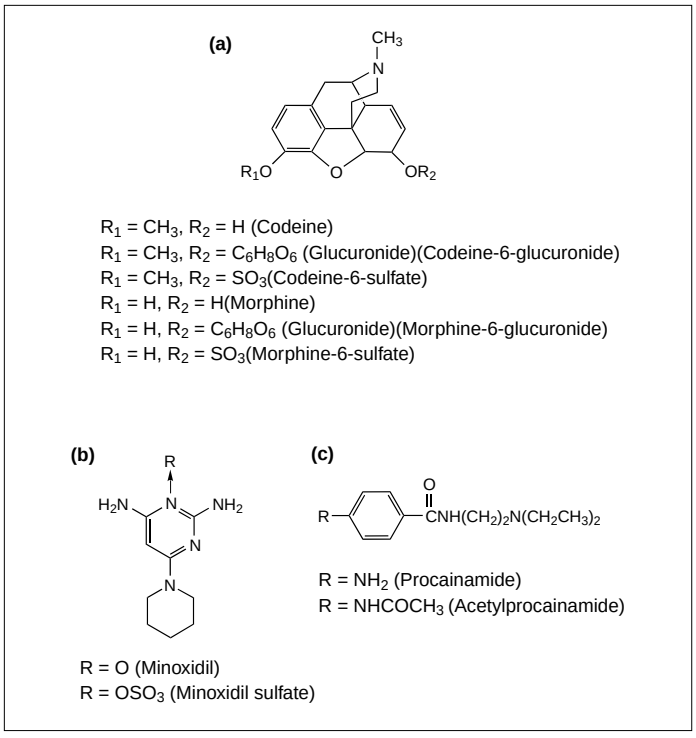

FIGURE 2

Examples of metabolites obtained through 'phase II' (conjugative) biotransformation reactions leading to pharmacological activation.

morphine (Figure 1d) [19]. *N*-demethylation of amitriptyline (an antidepressant) forms nortriptyline, an active metabolite that was developed as a drug in its own right (Figure 1e) [20]. Chlordiazepoxide (an anxiolytic drug) undergoes *N*-demethylation to a pharmacologically active desmethyl metabolite (Figure 1f) [21]. Oxidative deamination of chlordiazepoxide (Figure 1f) leads to the formation of an active ketone metabolite, demoxepam (Figure 1g) [21]. Other examples include the *S*-oxidation of thioridazine to mesoridazine, an antipsychotic drug that is twofold more potent in human compared with the parent drug (Figure 1h) [22]. Epoxidation of carbamazepine (an antiepileptic) (Figure 1i) leads to the formation of carbamazepine-10,11-epoxide, a stable active metabolite that might be responsible for most of the pharmacological activity of the drug (Figure 1j) [23]. Examples of biotransformation reactions that are mediated by phase II enzymes and form active metabolites include the 6-*O*-glucuronidation of morphine [24] and sulfation of morphine and codeine to form morphine-6-sulfate and codeine-6-sulfate, respectively [25] (Figure 2a), sulfation of minoxidil (antihypertensive agent and hair growth promoter) (Figure 2b) [26] and acetylation of procainamide (used for the treatment of premature ventricular contraction) (Figure 2c) [27].

As discussed thus far, pharmacologically active metabolites can be generated as primary, secondary, tertiary products and so on, in several consecutive steps often involving one or more enzyme systems. Multiple active metabolites can also be formed from a single compound. For example, tamoxifen, a drug used for the treatment of breast cancer, forms multiple active metabolites by undergoing CYP-mediated aromatic hydroxylation, *N*-demethylation and FMO-catalyzed *N*-oxidation, resulting in a 3-hydroxyltamoxifen (droloxifene), 4′- and 4-hydroxy tamoxifen, *N*-desmethyl tamoxifen and tamoxifen *N*-oxide, respectively (Figure 3a) [28–31]. The secondary metabolites, *N*-didesmethyltamoxifen and 4-hydroxy-desmethytamoxifen (endoxifen), are also pharmacologically active (Figure 3a) [30,32]. Table 1 contains more examples of drugs that, upon biotransformation reactions, result in the formation of active metabolites. Table 1 also illustrates modes of biotransformation reactions that result in the generation of the active metabolites. As shown in Table 1, these drugs belong to different chemical classes and are used in different areas of disease clusters.

Structure–activity relationships

Although metabolites are chemically distinct from the parent drug, they have structural similarities to the parent molecules that might help them, to some degree, attain activities similar to the parent drug. As illustrated above, this is particularly true for those biotransformations that involve simple functionalization reactions, such as hydroxylation, *O*- or *N*-demethylation and dehydrogenation or heteroatom oxidation, as is the case with many CYP-mediated reactions. Examples, in addition to those discussed above, are the simple hydroxylation or reduction or successive *N*-demethylation products of several antidepressant drugs that result in one or more active metabolites with significant therapeutic effect [18,33,34].

In general, if the functionalization reaction occurs at the auxophoric group (a non-pharmacophoric group) that is not crucial for the proper binding of the parent molecule to the receptor or enzyme, or if the reaction leads to optimization of the binding, it is very likely that the metabolite will maintain or enhance the potency of the parent compound. If, by contrast, the functionalization reactions lead to the formation of auxophoric groups that interfere with proper binding to the receptor or enzyme, or if the pharmacophoric group undergoes biotransformation, a decrease in activity is expected. In addition, a significant change in physicochemical properties or molecular size and shape can cause a significant loss in potency. An illustrative example is the biotransformation of tamoxifen to multiple metabolites that have different degrees of potency (Figure 3a). For example, the *N*-desmethyltamoxifen was found to have a lower binding affinity and antiestrogenic activity compared with the parent drug *in vitro*. Removal of the second methyl group led to the didesmethyl metabolite that has even lower antiestrogenic activity than the *N*-desmethyl metabolite. This is because of the successive demethylation from the side chain (the alkylaminoethoxy) that is important for the antiestrogenic activity of the drug. In fact, further removal of the whole dimethylaminoethane side chain results in the conversion of the molecule to a full agonist [35]. By contrast, the aromatic hydroxylation of the drug results in the formation of the 4-hydroxytamoxifen, a metabolite that is 30–100-fold more potent than the parent compound in terms of binding affinity to the estrogen receptor and in suppressing estrogen-dependent cell growth [28,30]. Interestingly, the secondary metabolite, *N*-desmethyl-4-hydroxytamoxifen (endoxifen), has shown estrogen receptor-binding affinity comparable with the 4-hydroxytamoxifen. Endoxifen has similar potency in suppressing estrogen dependent cell proliferation to 4-hydroxytamoxifen [30,32]. In this case, the partial loss in activity of tamoxifen as a result of demethylation has been overcompensated by the higher potency built in through the hydroxylation of the aromatic ring.

As described above, when metabolites have significant structural similarities to the parent molecules, it is not surprising that they

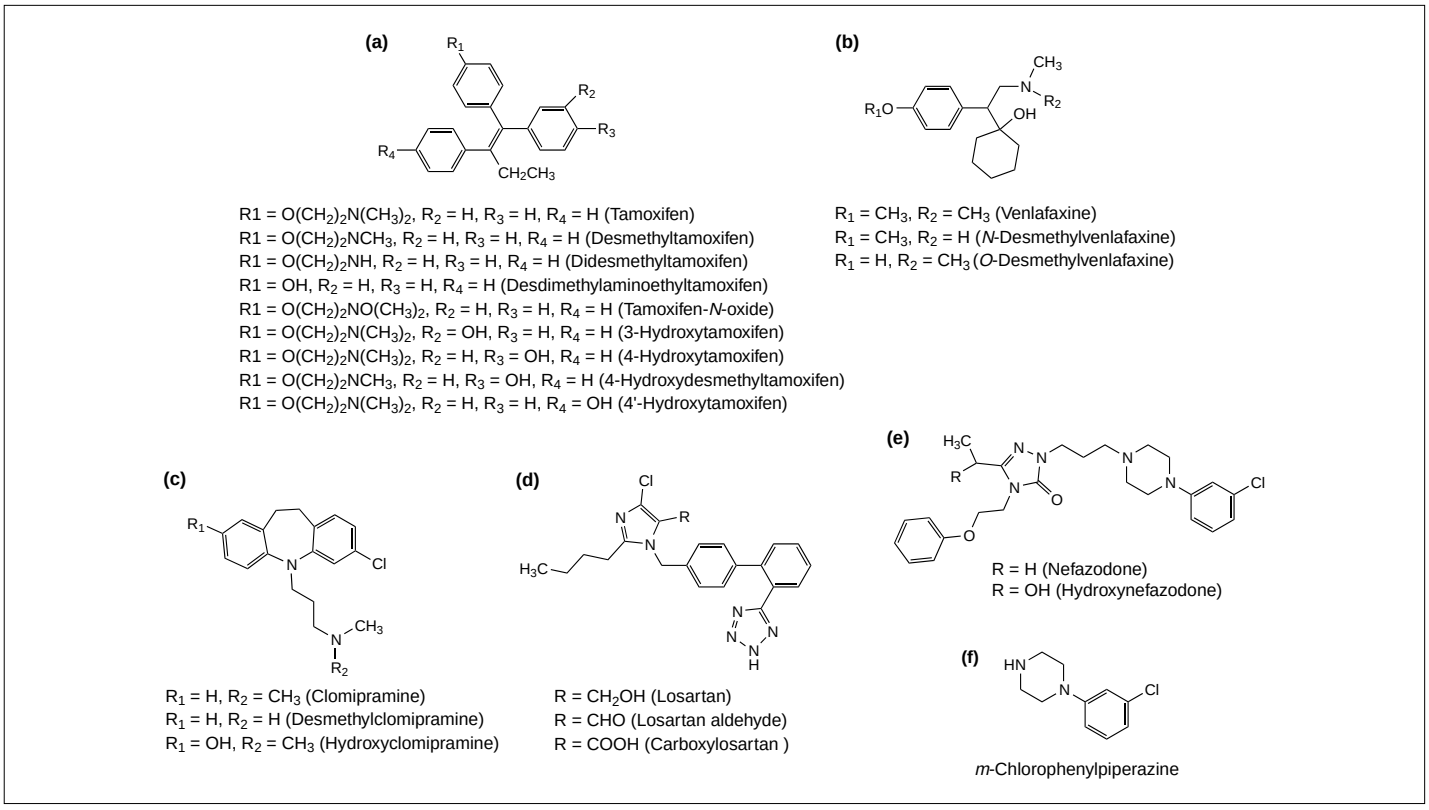

FIGURE 3

Examples of biotransformation reactions that result in the conversion of a parent drug to metabolites with broad range of pharmacological activities (on- or off-target).

have biochemical actions similar to their parents. However, a minor structural modification can result in loss of potency or modification of the biochemical mode of action of the parent drug. For example *O*-demethylation of venlafaxine (Figure 3b) leads to an active metabolite, *O*-desmethylvenlafaxine, which has similar pharmacology as the parent drug, whereas *N*-demethylation of venlafaxine results in an inactive metabolite [18]. Clomipramine (Figure 3c) is a potent inhibitor of serotonin reuptake, whereas its desmethyl metabolite selectively inhibits the reuptake of noradrenaline [33]. By contrast, the 8-hydroxy clomipramine metabolite inhibits serotonin reuptake similar to the parent drug. The metabolism of losartan (Figure 3d) results in the formation of an aldehyde metabolite that has angiotensin II receptor-independent anti-inflammatory and anti-aggregatory properties. Further metabolism of the aldehyde metabolite produces a carboxylic acid metabolite that is mainly responsible for the antihypertensive property of the drug through the antagonism of the angiotensin II receptor [36].

As is the case of desdimethylaminoethyl tamoxifen, biotransformation reactions that lead to significant structural modification of drugs will most likely result in loss of potency or a change in the biochemical action of drugs in an opposite or unrelated manner. For example, nefazodone (an antidepressant drug withdrawn recently from the market) undergoes hydroxylation at the ethyl side chain of the triazolone core to form hydroxynefazodone that has a similar *in vitro* pharmacological profile and is considered to have similar clinical efficacy to the parent drug (Figure 3e). By contrast, *N*-dealkylation at the piperazinyl nitrogen leads to the loss of a big portion of the molecule and forms *m*-chlorophenylpiperazine (Figure 3f), a metabolite that is an agonist at the serotonin 5-HT_{2C} receptor, whereas nefazodone or hydroxynefazodone are antagonists at this receptor [34,37].

Most metabolites that result from the major phase II drug metabolizing reactions, such as glucuronidation, are not pharmacologically active. This is presumably because these reactions entail a significant change in the physicochemical properties and molecular size and shape of the metabolites relative to the parent compounds. There are few examples of conjugated metabolites, such as morphine-6-*O*-glucuronide or morphine-6-sulfate, that are more potent than the parent drug [19,25].

Pharmacodynamic and pharmacokinetic aspects of active metabolites

Depending on the structural similarities and how well the bioactive conformation of the parent molecule is preserved or optimized during the biotransformation, metabolites can have a broad range of pharmacological activities. Consequently, potency, efficacy and the nature of the dose–response curve (flat, shallow or sharp) of an active metabolite relative to the parent are important determinants of the degree of its contribution to the overall therapeutic effect of the drug. For example, most of the pharmacological activity of primidone (an anticonvulsant drug that is no longer in clinical use) was believed to be caused by its active metabolite, phenobarbital. This is because phenobarbital, although formed as a minor metabolite of primidone, was much more potent than the parent drug [38].

The extent of contribution of active metabolites to the overall pharmacological action of drugs also depends on their pharmacokinetic characteristics (exposure and duration of action). Thus,

TABLE 1

Examples of drugs forming active metabolites and their mode of biotransformation

Drug	Metabolite	Mode of biotransformation	Clinical use	Refs
Acetohexamide	Hydroxyacetohexamide	Reduction of carbonyl to hydroxyl group	Antihyperglycemic agents	[59]
Diazepam	Desmethyldiazepam	*N*-demethylation	Tranquilizer and/or anticonvulsant	[60]
Dothiepin	Dothiepinsulfoxide	*S*-oxidation	Antidepressant	[34]
Fluoxetine	Norfluoxetine	*N*-demethylation	Antidepressant	[13]
Hydroxyzine	Cetirizine	Carboxylation	Antiallergic	[14,61]
Imipramine	Desimipramine	*N*-demethylation	Antidepressant	[62]
Loratadine	Desloratadine	Decarboethoxylation	Antiallergic	[63]
β-methyldigoxin	Digoxin	*O*-demethylation	Treatment for heart failure	[64]
Nalidixic acid	Hydroxynalidixic acid	Aliphatic hydroxylation	Antibacterial	[65]
Phenacetin	Acetaminophen	Deethylation	Analgesic	[66]
Prednisone	Prednisolone	Dehydrogenation	Used for the treatment of asthma	[67]
Proguanil	Cycloguanil	Cyclization	Antimalaria	[40]
Propafenone	5-hydroxypropafenone	Aromatic hydroxylation	Antiarrhythmic	[68]
Propranolol	4-hydroxypropranolol	Aliphatic hydroxylation	Antiarrhythmic	[69]
Risperidone	9-hydroxyrisperidone	Aliphatic hydroxylation	Antipsychotic	[70]
Simavastatin	6-β-hydroxymethyl-simavastatin	Aliphatic hydroxylation	Treatment of hypercholesterolemia	[11,12]
Terfenadine	Fexofenadine	Carboxylation	Antiallergic (antihistamine)	[14,15,71]
Verapamil	Norverapamil	*N*-demethylation	Arrhythmias, antianginal therapy and myocardial ischemia	[72]

the absorption, distribution (extravascular and vascular), metabolism and excretion characteristics of the metabolites are important parameters in determining whether a given biologically active metabolite is therapeutically significant. In most cases, metabolites are more polar and their protein binding, membrane permeability and tissue distribution might be different from those of the parent. Several metabolites are also metabolically more stable and have longer plasma elimination half-lives than the parents. For example, *N*-desmethyl active metabolites of several antidepressant drugs have plasma elimination half-lives about twofold longer than the parent drugs [34]. Metabolites with longer plasma elimination half-lives can lead to increased accumulation of the metabolites upon chronic dosing, even when they are formed as minor metabolites and their systemic concentrations upon acute dosing appear to be very low. The accumulation of active metabolites in plasma and tissues can lead to significant therapeutic or adverse effects and might necessitate dose adjustment.

Several drug metabolizing enzymes, such as CYP2C9, CYP2C19, CYP2D6 and *N*-acetyl transferase show genetic polymorphism. Thus, there are subsets of populations that are poor or fast drug metabolizers. If an active metabolite is formed by any of these polymorphically expressed enzymes and the active metabolite plays a significant role in the pharmacological activity of the drug, then chances are that pharmacological activity will be diminished in humans that are poor metabolizers. In such circumstances, dose adjustment is required. By contrast, ultrametabolizers of the parent drug will have a higher concentration of the active metabolites, possibly leading to supra-pharmacology and toxicity caused by the active metabolites. Examples of metabolic reactions catalyzed by polymorphic enzymes that give rise to active metabolites are: oxidation of losartan to carboxylosartan by CYP2C9 [39]; oxidation of proguanil to cycloguanil by CYP2C19 [40] and the CYP2D6-mediated *O*-demethylation of codeine to morphine [19]. The CYP2D6 poor metabolizers, for example, will experience reduced analgesia from the standard treatment with codeine. It is also possible to have active metabolites eliminated from the body by polymorphic enzymes, which might lead to changes in pharmacodynamic profile or potential adverse effects.

In general, blood flow to liver and liver size decrease in older people compared with young adults, which can lead to an age-related decline in hepatic drug clearance, which could be clinically important [41]. Concomitant with creatinine clearance, typically renal drug clearance also declines with age [42]. This is an important factor, as many drug metabolites tend to be metabolically more stable, more polar than the parent and cleared by the kidney. Accumulation of these active metabolites could occur when renal function is compromised. This change in pharmacokinetics of the parent and the active metabolites might necessitate dose adjustment in older patients. For example, the plasma elimination half-life of norfluoxetine, an active metabolite of fluoxetine, is ~7–15 days in young adults and ~20 days in geriatrics [13,42].

Other factors, such as interindividual variability, gender, diet and pathological conditions, could also affect the formation and clearance of active metabolites. For drugs that exhibit nonlinear pharmacokinetics, the ratio of active metabolite to parent can also change with dose as a result of saturable processes. Drug–drug interactions are also important and can greatly impact the formation and clearance of active metabolites. Such interactions can be caused by induction or inhibition of the drug-metabolizing enzymes. Induction and inhibition of enzymes can increase or decrease the concentration of active metabolites, depending on whether the induced or the inhibited enzyme is responsible for either the formation or clearance of the metabolites. The interaction might necessitate either a higher or lower dose than normal,

particularly if most of the efficacy upon the standard treatment is caused by the active metabolite or if the metabolite exhibits concentration-dependent toxicity.

Active metabolites themselves could induce or inhibit drug-metabolizing enzymes and as a result could also affect the pharmacokinetic profile of other drugs. For example, norfluoxetine, an active metabolite of fluoxetine, is a potent inhibitor of CYP2D6. In addition, as a result of the long half-life (up to two weeks) of the metabolite, the inhibitory effect persists for weeks after administration of fluoxetine has been discontinued [13].

Cellular membrane transporters can also have an impact on the disposition and elimination of active metabolites. For example, cetirizine, a drug in its own right, is a substrate of P-glycoprotein (P-gp), whereas its parent drug, hydroxyzine, is not [43]. This is presumably the reason for the restricted distribution of cetirizine to brain tissue and, hence, for its lack of sedative properties compared with the parent drug. Losartan is a P-gp substrate, but its carboxylic acid active metabolite is not [44]. Verapamil and its active metabolite are P-gp inhibitors, whereas the other *N*-dealkylated metabolites of verapamil are not inhibitors but substrates of P-gp [45].

The significance of active metabolites in drug discovery and development

There are several reasons why it is important to be aware of the significance of pharmacologically active metabolites. Described below are some of the roles that active metabolite might play at the various stages of drug discovery (e.g. lead compound selection and optimization) and development.

Active metabolites as lead candidates during lead optimization

Pharmacologically active metabolites can be conveniently used as leads where they are subjected to further structural modification to obtain improved lead candidates during the lead optimization phase of drug discovery. Such approach was used, for example, in the discovery of ezetimibe, a cholesterol absorption inhibitor [46–48]. In this work, an active metabolite that was already ~30-fold more potent than the parent was further modified to give the final drug candidate (ezetimibe), which was 400-fold more potent than the original lead. This approach can also be effectively exploited when a given chemotype in a drug discovery program suffers from issues such as solubility or metabolic stability. As discussed previously, metabolites generally have improved aqueous solubility or metabolic stability compared with their corresponding parent molecules. Thus, if during the lead optimization stage a metabolite with sufficient biological activity is identified, the metabolite can be used as a lead with an added advantage of improved properties and might be optimized further.

Active metabolites as drugs in their own right

If an active metabolite has improved pharmacological, pharmacokinetic and toxicological properties compared with the parent, it should be advanced to the clinic in its own right as a viable drug candidate. In this regard, there are several active metabolites on the market that have already been developed as drugs and some examples are listed in Table 2. For some of these drugs, only the metabolite forms (e.g. acetaminophen, fexofenadine and phenobarbital) are currently in clinical use. As discussed in a recent review [8], there is a lot of potential in developing an active metabolite as a drug in terms of improved pharmacodynamic, pharmacokinetic and safety profiles in comparison with the parent compound. A good example of this is the metabolism of terfenadine to its active metabolite, fexofenadine by CYP3A4. In the presence of CYP3A4 inhibitor, the drug was found to cause severe cardiotoxicity and consequently was withdrawn from the market and replaced by fexofenadine [49]. Therefore, whenever possible, an effort should be made to characterize an active metabolite in terms of intrinsic potency, bioavailability, rate of clearance, tissue distribution and safety to determine whether the metabolite can be a viable clinical candidate. Moreover, timely discovery of active metabolites would enable the parent molecule to have more complete patent protection.

TABLE 2

Active metabolites developed as drugs

Metabolite drugs	Parent drugs
Acetaminophen	Phenacetin
Cetirizine	Hydroxyzine
Desimipramine	Imipramine
Desloratadine	Loratadine
Digoxin	β-methyldigoxin
Fexofenadine	Terfenadine
Mesoridazone	Thioridazone
Morphine	Codeine
Nortriptyline	Amitriptyline
Oxazepam	Diazepam
Phenobarabital	Primidone

Placing preclinical and clinical pharmacological and pharmacokinetic data in perspective

If an active metabolite has a significant potency or an improved pharmacokinetic profile, it is very likely that there is a greater and/or longer duration of pharmacological action than is expected from the corresponding *in vitro* activity or pharmacokinetic data of the parent compound during preclinical studies. Thus, a timely recognition of the role of active metabolites enables the understanding of the observed *in vivo* pharmacodynamic data that might not be apparent from the *in vitro* activity and pharmacokinetics of the parent drug. Similarly, in clinical studies where the parent drug exhibits unexpectedly enhanced *in vivo* pharmacological activity, identification of active metabolites is important to understand the underlying cause and to come up with a better study design.

Extrapolating the pharmacodynamic data observed in the preclinical animal model to human

It is known that differences can exist among species in the rate and extent of drug metabolism, with quantitative differences being more common than qualitative ones. As a result, the nature (qualitative) and amount (quantitative) of active metabolites formed in an experimental animal model and their relative contribution to the overall pharmacological activity can be very different from those in human. For example, dogs are unable to acetylate *N*-arylamines, therefore active metabolites such as acetyl procainamide

are not formed in dogs; cats are unable to glucuronidate compounds that are well known to undergo glucuronidation in other species including human. In rats and mice only the inactive metabolite of morphine-3-*O*-glucuronide is produced, whereas in human both the morphine-3-*O*-glucuronide and the 6-*O*-glucuronide (the active metabolite) are formed [24]. The plasma AUC (area under the plasma concentration–time curve) ratios of hydroxybupropion (an active metabolite) to bupropion in mice, rats, dogs and humans were ~3, 0.3, 1, 16, respectively, after oral administration of bupropion, clearly indicating significant species differences in the extent of formation of the active metabolite [50]. This was also consistent with the differences seen in the pharmacology of the compound in rats and mice. Oral administration of bupropion prevented tetrabenzine-induced sedation in mice but not in rats [50].

Therefore, to improve the decision making process at the discovery stage and to design appropriate clinical studies early on, it is important to appreciate the considerable differences between species in terms of rate of formation and clearance of metabolites. Under these circumstances, it is recommended that a correlation of *in vitro* and *in vivo* metabolism and pharmacodynamic data are established in the experimental animal model and these data are used in combination with the human *in vitro* metabolism systems to project human pharmacokinetics and efficacious dose.

Understanding the mechanism of action of drugs

In most cases, active metabolites, particularly those formed as primary metabolites, have biochemical actions similar to their parents. However, an active metabolite can also have biochemical actions that are synergistic or inhibitory to the action of the parent drug or act on off-target pharmacology that is completely unrelated to that of the parent drug. Recognition and appreciation of the role of active metabolites is thus important to fully understand and tease out the mechanism of action of drugs early on.

Recognizing and predicting any pharmacology mechanism-based toxicity mediated by active metabolites

Appreciation of the presence of active metabolite is important to understand any adverse effect that might arise from supra-pharmacology during either preclinical or clinical studies and to make an appropriate dose adjustment to mitigate the toxicity in the clinic. If a parent drug shows mechanism-based toxicity, then one would also expect pharmacologically active metabolites to exhibit similar effects. In fact, the effect can be even more pronounced if the active metabolite is relatively more potent and has higher free fraction and long pharmacokinetic half-life, which leads to accumulation. For example, accumulation of the active metabolites of tolazamide, an antihyperglycemic agent, is considered to be the cause for the recurrent hypoglycemic coma observed after treatment of patients with this drug [51]. Another example is the clinical incidence observed with flosequinan, an ionotropic and vasodilator drug developed for the treatment of heart failure. The high mortality rate observed after treatment with this drug is believed to be caused by an exaggerated pharmacology of its active metabolite, flosequinoxan. Flosequinoxan, a sulfone metabolite of flosquinan, is slightly more potent than the parent compound and was shown to have a higher steady-state plasma concentration and a much longer plasma elimination half-life compared with the parent compound [52,53]. The supra-pharmacological effect can become even more crucial if the clearance and formation rate of the metabolite is modulated by drug–drug interaction or genetic variability.

Experimental indicators of the presence and involvement of active metabolites

There are several experimental observations that can be used as indicators of the presence and involvement of active metabolites. Some of them are discussed below.

Apparent disconnect between in vitro *and* in vivo *pharmacological data*

Because of the presence of active metabolites, a greater pharmacological response can be observed that is inconsistent with *in vitro* biological data. For example, because of the contribution of active metabolites, significant differences might be observed *in vivo* in the animal model of choice among several compounds of the same chemical class, despite the fact that the compounds exhibit similar *in vitro* activity and a similar pharmacokinetic profile (e.g. plasma half-life and tissue distribution) [8].

Apparent disconnect between pharmacodynamic and pharmacokinetic data

When a compound with a short pharmacokinetic half-life shows unexpectedly prolonged pharmacological action, it can be indicative of the presence of active metabolite(s). For example, the pharmacodynamic half-life of atorvastatin is twofold longer than the pharmacokinetic half-life of the compound because of the contribution of its active metabolites [10].

Higher pharmacological response for compounds given extravascularly versus parenterally

If a parent compound undergoes extensive first-pass metabolism upon oral administration, its bioavailability and consequently its plasma concentration will be lower than the plasma concentration obtained, for example, via an intravenous route. However, the concentration of the metabolites following oral administration will be higher (with respect to intravenous administration). Therefore, under these conditions, there is a strong indication that active metabolites are involved, if a higher pharmacological response is observed after oral administration than after intravenous administration. Of course, this assumes that the first-pass organs are not the site of the pharmacological action. In addition, it might be necessary to adjust the doses to make sure that parent drug concentrations are independent of dose route.

Higher or lower pharmacological response in the presence of metabolism modulators

The presence of active metabolites might be inferred if an interaction with concomitantly administered drugs (inhibitors or inducers) or, for example, a genetic polymorphism results in: (i) a decrease of pharmacological response in spite of an increase in the systemic concentration of the parent; (ii) an increase in pharmacological response despite a decrease in the systemic concentration of the parent or (iii) either an increase or decrease in pharmacological activity, even when there is no change in the corresponding pharmacokinetic profile of the parent. In this instance, particularly

at the discovery stage, biotransformation of parent can be blocked by using a suitable inhibitory agent, such as 1-aminobenzotriazole, or an antifungal drug, such as clotrimazole. Inhibitory agents will increase parent drug levels, which contribute to increased pharmacological activity, therefore, such studies have to be conducted carefully.

Unintended pharmacological responses

If a compound shows an additional pharmacological response *in vivo* unrelated to or opposite to that expected from its interaction with a proven or established pharmacological target, it might be caused by active metabolites. For example, losartan was developed as an antihypertensive drug through the antagonism of the angiotensin II receptor, mainly by the action of its active carboxylic acid metabolite. However, the observation that the compound also had anti-inflammatory and anti-aggregatory properties independent of the angiotensin II receptor antagonism led to the identification of another (aldehyde) active metabolite that was largely responsible for this additional pharmacological action [36].

Approaches used to assess pharmacological activity of metabolites

The presence and impact of active metabolites can be evaluated and confirmed using the following approaches.

In silico *screening*

Virtual screening can be used to assess the biological activity and binding affinity of active metabolites with commonly used computational techniques, such as ligand-based pharmacophore modeling and structure-based docking, which might have already been developed for the purpose of discovering or optimizing the parent compound. Ligand-based modeling can be built on existing knowledge of the biological activity of the parent molecule and/or other compounds with known activity against the pharmacological target of interest, whereas structure-based docking can utilize the 3D structure of the binding sites of the target (receptor or enzyme) of interest. For example, a search for molecular homology of metabolites of losartan to known anti-inflammatory drugs was used in the identification of an active metabolite with anti-inflammatory and anti-aggregatory properties [36].

In vitro *screening*

Metabolites can be tested for their biological activity by using targeted screening approach against a particular pharmacological target in high or medium throughput mode. This is particularly useful if classes of compounds within a drug discovery program are known to undergo extensive *in vitro* metabolism. In this instance, it is possible to generate sufficient quantities of metabolites more conveniently and in a timely fashion by using various biological methods described below. Their pharmacological activity can be tested either as a mix, before or after isolation of the metabolites [8].

In vivo *activity studies*

In vivo pharmacological methods are used to assess and establish the actual contribution of the metabolite to the overall therapeutic outcome. This can be done, for example, by comparing the overall pharmacodynamic and pharmacokinetic profile of the metabolite and the parent compound. The actual contribution of any active metabolite to the overall pharmacological action of a drug can be unequivocally ascertained by dosing the metabolite itself *in vivo* via an appropriate route of administration. Exceptions to this are metabolites that either need to reach the site of action in the parent form, where they are released via pertinent biotransformation reactions, or metabolites with *in vivo* dispositions influenced by the presence of the parent. The direct dosing of the metabolite enables one to obtain pharmacokinetic and tissue distribution data for the metabolite. The resulting data can be used to determine whether the metabolite can be developed as a drug in its own right. Using appropriate study design, the data might also aid the assessment of any off-target pharmacological activity.

Generation and structural characterization of active metabolites

Various biological or chemical methods can be utilized to generate active metabolites and to assess their *in vitro* and *in vivo* biological activity. Biological methods include incubation of the parent compound with various subcellular fractions (e.g. microsomes), individual recombinant drug-metabolizing enzymes, whole cells (e.g. hepatocytes) or tissue slices [8,16]. *In vivo* samples such as bile, urine and plasma collected from experimental animals and humans following administration of the parent compound can also be the source of active metabolites. When large scale synthesis is required to perform *in vivo* studies or to manipulate further the metabolites for lead optimization purpose, microbial or chemical synthetic methods can be used [8].

Modern analytical techniques such as LC–MS–MS, LC–NMR and LC–NMR–MS are now available to structurally characterize the desired metabolites in a timely fashion [54–57]. These techniques have the capability of online analysis combined with great sensitivity, specificity and speed. For example, it is now possible to obtain qualitative information on the metabolites while simultaneously acquiring quantitative information on the parent in *in vivo* and *in vitro* samples [58].

Conclusions

Several drugs are biotransformed to pharmacologically active metabolites that can significantly contribute to the overall pharmacological or adverse effects of drugs. For the most part, the pharmacological actions attributed to active metabolites are similar to those of the parent. However, active metabolites could also mediate therapeutic effects that are synergistic or even the reverse of the pharmacological action of the parent drugs. They could also mediate pharmacological actions that are unrelated to that of the parent compound. Therefore, to fully understand the mechanism of action of drugs, it is imperative to recognize the role of active metabolites in drug discovery and development.

Recognition of the role of active metabolites early on will enable the correct interpretation of pharmacodynamic data observed in a particular preclinical animal model and extrapolation of the data to human. Moreover, at the discovery stage, active metabolites can be used as leads that could be subjected to further structural modifications during the lead optimization phase. In addition, active metabolites might exhibit superior druggability profiles compared with the parent molecules and therefore be developed as drugs in their own right.

There are several experimental observations or *in silico* findings that can be used as indicators for the presence and involvement of

active metabolites. There are also several approaches that can be taken to evaluate the impact of active metabolites on the overall pharmacological action of drugs.

For any active metabolite, the extent of its contribution to the overall therapeutic and adverse effect profile depends on its relative intrinsic potency and pharmacokinetic properties. It is therefore important to recognize the effect of various factors that govern the exposure to such metabolites (e.g. drug–drug interactions, interindividual variability, genetic differences, environment and age).

Acknowledgements

The author gratefully acknowledges Dr David Rodrigues and Dr William Humphreys for their critical review of this manuscript.

References

1 Kumar, G.N. and Surapaneni, S. (2001) Role of drug metabolism in drug discovery and development. *Med. Res. Rev.* 21, 397–411
2 White, R.E. (2000) High-throughput screening in drug metabolism and pharmacokinetic support of drug discovery. *Annu. Rev. Pharmacol. Toxicol.* 40, 133–157
3 White, R.E. (1998) Short- and long-term projections about the use of drug metabolism in drug discovery and development. *Drug Metab. Dispos.* 26, 1213–1216
4 Baillie, T.A. *et al.* (2002) Drug metabolites in safety testing. *Toxicol. Appl. Pharmacol.* 182, 188–196
5 Guengerich, F.P. (2001) Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem. Res. Toxicol.* 14, 611–650
6 Park, B.K. *et al.* (2005) The role of metabolic activation in drug-induced hepatotoxicity. *Annu. Rev. Pharmacol. Toxicol.* 45, 177–202
7 Walgren, J.L. *et al.* (2005) Role of metabolism in drug-induced idiosyncratic hepatotoxicity. *Crit. Rev. Toxicol.* 35, 325–361
8 Fura, A. *et al.* (2004) Discovering drugs through biological transformation: role of pharmacologically active metabolites in drug discovery. *J. Med. Chem.* 47, 4339–4351
9 Garattini, S. (1985) Active drug metabolites: an overview of their relevance in clinical pharmacokinetics. *Clin. Pharmacokinet.* 10, 216–227
10 Lennernas, H. (2003) Clinical pharmacokinetics of atorvastatin. *Clin. Pharmacokinet.* 42, 1141–1160
11 Williams, D. and Feely, J. (2002) Pharmacokinetic-pharmacodynamic drug interactions with HMG-CoA reductase inhibitors. *Clin. Pharmacokinet.* 41, 343–370
12 Prueksaritanont, T. *et al.* (2003) The human hepatic metabolism of simvastatin hydroxy acid is mediated primarily by CYP3A, and not CYP2D6. *Br. J. Clin. Pharmacol.* 56, 120–124
13 Cheer, S.M. and Goa, K.L. (2001) Fluoxetine: A review of its therapeutic potential in the treatment of depression associated with physical illness. *Drugs* 61, 81–110
14 Golightly, L.K. and Greos, L.S. (2005) Second-generation antihistamines: actions and efficacy in the management of allergic disorders. *Drugs* 65, 341–384
15 Meeves, S.G. and Appajosyula, S. (2003) Efficacy and safety profile of fexofenadine HCl: a unique therapeutic option in H1-receptor antagonist treatment. *J. Allergy Clin. Immunol.* 112, S69–S77
16 Nassar, A.FE *et al.* (2004) Improving the decision-making process in the structural modification of drug candidates: enhancing metabolic stability. *Drug Discov. Today* 9, 1020–1028
17 Shet, M.S. *et al.* (1997) Metabolism of the antiandrogenic drug (Flutamide) by human CYP1A2. *Drug Metab. Dispos.* 25, 1298–1303
18 Rotzinger, S. *et al.* (1999) Metabolism of some 'second'- and 'fourth'-generation antidepressants: iprindole, viloxazine, bupropion, mianserin, maprotiline, trazodone, nefazodone, and venlafaxine. *Cell. Mol. Neurobiol.* 19, 427–442
19 Lotsch, J. *et al.* (2002) Drug interactions with patient-controlled analgesia. *Clin. Pharmacokinet.* 41, 31–57
20 Ulrich, S. and Lauter, J. (2002) Comprehensive survey of the relationship between serum concentration and therapeutic effect of amitriptyline in depression. *Clin. Pharmacokinet.* 41, 853–876
21 Greenblatt, D.J. *et al.* (1978) Pharmacokinetics of chlordiazepoxide and metabolites following single and multiple oral doses. *Int. J. Clin. Pharmacol. Biopharm.* 16, 486–493
22 Llerena, A. *et al.* (2000) Use of the mesoridazine/thioridazine ratio as a marker for CYP2D6 enzyme activity. *Ther. Drug Monit.* 22, 397–401
23 Potter, J.M. and Donnelly, A. (1998) Carbamazepine-10,11-epoxide in therapeutic drug monitoring. *Ther. Drug Monit.* 20, 652–657
24 Ritter, J.K. (2000) Roles of glucuronidation and UDP-glucuronosyltransferases in xenobiotic bioactivation reactions. *Chem. Biol. Interact.* 129, 171–193
25 Zuckerman, A. *et al.* (1999) Pharmacological characterization of morphine-6-sulfate and codeine-6-sulfate. Pharmacological characterization of morphine-6-sulfate and codeine-6-sulfate. *Brain Res.* 842, 1–5
26 Anderson, R.J. *et al.* (1998) Sulfation of minoxidil by multiple human cytosolic sulfotransferases. *Chem. Biol. Interact.* 109, 53–67
27 Okumura, K. *et al.* (1997) Genotyping of N-acetylation polymorphism and correlation with procainamide metabolism. *Clin. Pharmacol. Ther.* 61, 509–517
28 Kemp, J.V. *et al.* (1983) Identification and biological activity of tamoxifen metabolites in human serum. *Biochem. Pharmacol.* 32, 2045–2052
29 Loser, R. *et al.* (1985) No loss of estrogenic or anti-estrogenic activity after demethylation of droloxifene (3-OH-tamoxifen). *Int. J. Cancer* 36, 701–703
30 Desta, Z. *et al.* (2004) Comprehensive evaluation of tamoxifen sequential biotransformation by the human cytochrome P450 system *in vitro*: prominent roles for CYP3A and CYP2D6. *J. Pharmacol. Exp. Ther.* 310, 1062–1075
31 Parte, P. and Kupfer, D. (2005) Oxidation of tamoxifen by human FMO1 and FMO3 to tamoxifen-N-oxide and its novel reduction back to tamoxifen by human cytochrome P450s and hemoglobin. *Drug Metab. Dispos.* 33, 1446–1452
32 Johnson, M.D. *et al.* (2004) Pharmacological characterization of 4-hydroxy-N-desmethyl tamoxifen, a novel active metabolite of tamoxifen. *Breast Cancer Res. Treat.* 85, 151–159
33 Rudorfer, M.V. and Potter, W.Z. (1997) The role of metabolites of antidepressants in the treatment of depression. *CNS Drugs* 7, 273–312
34 Sánchez, C. and Hyttel, J. (1999) Comparison of the effects of antidepressants and their metabolites on reuptake of biogenic amines and on receptor binding. *Cell. Mol. Neurobiol.* 19, 467–489
35 Jordan, V.C. and Gosden, B. (1982) Importance of the alkylaminoethoxy side-chain for the estrogenic and antiestrogenic actions of tamoxifen and trioxifene in the immature rat uterus. *Mol. Cell. Endocrinol.* 27, 291–306
36 Krämer, C. *et al.* (2002) Angiotensin II receptor-independent antiinflammatory and antiaggregatory properties of losartan: role of the active metabolite EXP3179. *Circ. Res.* 90, 770–776
37 Rotzinger, S. and Baker, G.B. (2002) Human CYP3A4 and the metabolism of nefazodone and hydroxynefazodone by human liver microsomes and heterologously expressed enzymes. *Eur. Neuropsychopharmacol.* 12, 91–100
38 Eadie, M.J. (1991) Formation of active metabolites of anticonvulsant drugs. A review of their pharmacokinetic and therapeutic significance. *Clin. Pharmacokinet.* 21, 27–41
39 Schmidt, B. and Schieffer, B. (2003) Angiotensin II AT1 receptor antagonist. Clinical implications of active metabolites. *J. Med. Chem.* 46, 2261–2270
40 Coller, J.K. *et al.* (1997) Association between CYP2C19 genotype and proguanil oxidative polymorphism. *Br. J. Clin. Pharmacol.* 43, 659–660
41 Wynne, H. (2005) Drug metabolism and ageing. *J. Br. Menopause Soc.* 11, 51–56
42 Cusack, B.J. (2004) Pharmacokinetics in older persons. *Am J Geriatr Pharmacother* 2, 274–302
43 Polli, J.W. *et al.* (2003) P-glycoprotein influences the brain concentrations of cetirizine (Zyrtec®), a second-generation non-sedating antihistamine. *J. Pharm. Sci.* 92, 2082–2089
44 Soldner, A. *et al.* (2000) Active transport of the angiotensin-II antagonist losartan and its main metabolite EXP 3174 across MDCK-MDR1 and Caco-2 cell monolayers. *Br. J. Pharmacol.* 129, 1235–1243
45 Pauli-Magnus, C. *et al.* (2000) Characterization of the major metabolites of verapamil as substrates and inhibitors of P-glycoprotein. *J. Pharmacol. Exp. Ther.* 293, 376–382
46 Clader, J.W. (2004) The Discovery of Ezetimibe: a view from outside the receptor. *J. Med. Chem.* 47, 1–9
47 Rosenblum, S.B. *et al.* (1998) Discovery of 1-(4-Fluorophenyl)-(3R)-[3-(4-fluorophenyl)-(3S)-hydroxypropyl]-(4S)-(4-hydroxyphenyl)-2-azetidinone (SCH 58235): a designed, potent, orally active inhibitor of cholesterol absorption. *J. Med. Chem.* 41, 973–980
48 Van Heek, M. *et al.* (1997) *In vivo* metabolism-based discovery of a potent cholesterol absorption inhibitor, SCH 58235, in the rat and rhesus monkey through identification of the active metabolites of SCH 48461. *J. Pharmacol. Exp. Ther.* 283, 157–163
49 Honig, P.K. *et al.* (1993) Terfenadine-ketoconazole interaction. Pharmacokinetic and electrocardiographic consequences. *JAMA* 269, 1513–1518
50 Welch, R.M. *et al.* (1987) Pharmacological significance of the species differences

in bupropion metabolism. *Xenobiotica* 17, 287–298
51 Harrower, A.D.B. (1996) Pharmacokinetics of oral antihyperglycaemic agents in patients with renal insufficiency. *Clin. Pharmacokinet.* 31, 111–119
52 Gallo, B.V. (1993) Pharmacokinetic profile of flosequinan in patients with compromised renal function. *J. Pharm. Sci.* 82, 282–285
53 Kamali, F. and Edwards, C. (1995) Possible role of metabolite in flosequinan-related mortality. *Clin. Pharmacokinet.* 29, 396–403
54 Watt, A.P. *et al.* (2003) Metabolite identification in drug discovery. *Curr. Opin. Drug Discov. Devel.* 6, 57–65
55 Kostiainen, R. *et al.* (2003) Liquid chromatography/atmospheric pressure ionization-mass spectrometry in drug metabolism studies. *J. Mass Spectrom.* 38, 357–372
56 Lindon, J.C. *et al.* (1996) The Development and application of coupled HPLC–NMR spectroscopy. *Adv. Chromatogr.* 36, 315–382
57 Corcoran, O. and Spraul, M. (2003) LC–NMR–MS in drug discovery. *Drug Discov. Today* 8, 624–31
58 Li, A.C. *et al.* (2005) Simultaneously quantifying parent drugs and screening for metabolites in plasma pharmacokinetic samples using selected reaction monitoring information-dependent acquisition on a QTrap instrument. *Rapid Commun. Mass Spectrom.* 19, 1943–1950
59 Imamura, Y. *et al.* (2001) Hypoglycemic effect of S(–)-hydroxyhexamide, a major metabolite of acetohexamide, and its enantiomer R(+)-hydroxyhexamide. *Life Sci.* 69, 1947–1955
60 Gall, M. *et al.* (1978) Pharmacology of some metabolites of triazolam, alprazolam, and diazepam prepared by a simple, one-step oxidation of benzodiazepines. *J. Med. Chem.* 21, 1290–1294
61 Gengo, F.M. *et al.* (1987) The relative antihistaminic and psychomotor effects of hydroxyzine and cetirizine. *Clin. Pharmacol. Ther.* 42, 265–272
62 Lemoine, A. *et al.* (1993) Major pathway of imipramine metabolism is catalyzed by cytochromes P-450 1A2 and P-450 3A4 in human liver. *Mol. Pharmacol.* 43, 827–832
63 Yumibe, N. *et al.* (1996) Identification of human liver cytochrome P450 enzymes that metabolize the nonsedating antihistamine loratadine: formation of descarboethoxyloaratadine by CYP3A4 and CYP2D6. *Biochem. Pharmacol.* 51, 165–172
64 Rietbrock, N. *et al.* (1975) Disposition of beta-methyldigoxin in man. *Eur. J. Clin. Pharmacol.* 9, 105–114
65 Ferry, N. *et al.* (1981) Nalidixic acid kinetics after single and repeated oral doses. *Clin. Pharmacol. Ther.* 29, 695–698
66 Belle, D.J. *et al.* (2000) A population approach to enzyme characterization and identification: application to phenacetin O-deethylation. *Pharm. Res.* 17, 1531–1536
67 Jeng, S. *et al.* (2003) Prednisone metabolism in recipients of kidney or liver transplants and in lung recipients receiving ketoconazole. *Transplantation* 75, 792–795
68 Cahill, S.A. and Gross, G.J. (2004) Propafenone and its metabolites preferentially inhibit I_{Kr} in rabbit ventricular myocytes. *J. Pharmacol. Exp. Ther.* 308, 59–65
69 Albani, F. *et al.* (1984) Plasma concentrations of propranolol and 4-hydroxy-propranolol at steady-state in neurological patients: intersubject and intrasubject correlations with dose. *Int. J. Clin. Pharmacol. Res.* 4, 19–23
70 Knegtering, R. *et al.* (2005) Predominant role of the 9-hydroxy metabolite of risperidone in elevating blood prolactin levels. *Am. J. Psychiatry* 162, 1010–1012
71 Renwick, A.G. (1999) The metabolism of antihistamines and drug interactions: the role of cytochrome P450 enzymes. *Clin. Exp. Allergy* 29, 116–124
72 Wang, Y.H. *et al.* (2004) Prediction of cytochrome P450 3A inhibition by verapamil enantiomers and their metabolites. *Drug Metab. Dispos.* 32, 259–266