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Going beyond common drug metabolizing enzymes: Case studies of biotransformation involving aldehyde oxidase, gamma-glutamyl transpeptidase, cathepsin B, flavin-containing monooxygenase, and ADP-ribosyltransferase

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ABBREVIATIONS: ADC, antibody drug conjugate; ADR, adenine dinucleotide ribose; AO, aldehyde oxidase; ART, ADP-ribosyltransferase, BNPP, bis-(p-nitrophenyl) phosphate; BTK, Bruton's tyrosine kinase; CES, carboxylesterase; DCPIP, 2,6-dichlorophenolindophenol; FAD, flavin adenine dinucleotide; FMO, flavin-containing monooxygenase; GGT, gamma-glutamyl transpeptidase; GSH, glutathione; MoCo, molybdenum cofactor; NAC, *N*-acetylcysteine; NAD^+/H, nicotinamide adenine dinucleotide and its reduced form, $NADP^+$/H, nicotinamide adenine dinucleotide phosphate and its reduced form, NMN, nicotinamide mononucleotide; PAB, para-aminobenzyl; Val-Cit, valine-citrulline.

Abstract

The significant roles that cytochrome P450 (P450) and UDP-glucuronosyl transferase (UGT) enzymes play in drug discovery cannot be ignored, and these enzyme systems are commonly examined during drug optimization using liver microsomes or hepatocytes. At the same time, other drug metabolizing enzymes have a role in the metabolism of drugs and can lead to challenges in drug optimization that could be mitigated if the contributions of these enzymes were better understood. This paper presents examples (mostly from Genentech) of five different non-P450 and non-UGT enzymes that contribute to the metabolic clearance or bioactivation of drugs and drug candidates. Aldehyde oxidase mediates a unique amide hydrolysis of GDC-0834, leading to high clearance of the drug. Likewise, the rodent-specific ribose conjugation by ADP-ribosyltransferase leads to high clearance of an interleukin-2-inducible T-cell kinase inhibitor. Metabolic reactions by flavin-containing monooxygenases (FMO) are easily mistaken for P450-mediated metabolism such as oxidative defluorination of 4-fluoro-*N*-methylaniline by FMO. Gamma-glutamyl transpeptidase is involved in the initial hydrolysis of glutathione metabolites, leading to formation of proximate toxins and nephrotoxicity, as is observed with cisplatin in the clinic, or renal toxicity, as is observed with efavirenz in rodents. Finally, cathepsin B is a lysosomal enzyme that is highly expressed in human tumors and has been targeted to release potent cytotoxins, as in the case of brentuximab vedotin. These examples of non-P450- and non-UGT-mediated metabolism show that a more complete understanding of drug metabolizing enzymes allows for better insight into the fate of drugs and improved design strategies of molecules in drug discovery.

Introduction

The drug metabolism and pharmacokinetic disciplines play central roles in our understanding of the fate of most drugs in the body. Since most marketed drugs are cleared by metabolism (Williams, 2004), it is not surprising that a clear understanding of metabolism and metabolic enzymes and pathways are critical in drug discovery in order to optimize metabolic rates. The roles of cytochrome P450 (P450) and UDP-glucuronosyl transferase (UGT) enzymes are well established and considered the most important enzymes for metabolism of most drugs. While we do a good job with P450-mediated metabolism and predicting human metabolism via this pathway, other enzymes are less studied and tools are not readily available. Because the need for more creative drug design for more difficult to treat diseases, the broad metabolic stability screening and metabolite identification in early discovery using microsomal fractions or hepatocytes for oxidative and glucuronic acid-conjugative metabolism in the pharmaceutical industries has selected compounds as non-substrates of cytochrome P450s or UGTs as new drug candidates. This strategy would ultimately lead to discovery of the new chemical entities (NCE) falling in chemical spaces which are substrates of non-P450 or non-UGT enzymes for their biotransformation and disposition. The role of non-P450 oxidation enzymes in drug biotransformation has been recognized in recent years. This review will use selected examples mainly from Genentech to illustrate metabolism catalyzed by some of these enzymes, namely, aldehyde oxidase and flavin-containing monooxygenase as non-P450 oxidation enzymes, ADP-ribosyltransferase as a non-UGT conjugation enzyme, and gamma-glutamyl transpeptidase, and cathepsin B as hydrolysis enzymes. The two hydrolysis enzymes were selected to reflect the increased importance of prodrugs and antibody drug conjugates as expanded new chemical spaces. Although hydrolysis-mediated bioactivation was intended for drug release from pro-drugs and antibody drug conjugates (ADC) to support their pharmacological activities, detailed understanding on hydrolytic enzyme kinetics and substrate specificity as well as non-target tissue expression is an important area of research for optimizations of prodrugs and next generation of ADCs.

Aldehyde oxidase (AO)

AO is a molybdo-flavoenzyme that is widely studied for its role in phase I metabolism of a number of xenobiotics in humans (Kitamura et al., 2006; Garattini and Terao, 2012). This enzyme in humans has only one isoform (AOX1) and is known to be involved in both oxidation and reduction metabolic reactions (Pryde DC et al., 2010). Recent studies have discovered that AO is also capable of catalyzing amide hydrolysis in human liver cytosol (Sodhi et al., 2015).

This discovery began with exploratory clinical studies that were conducted on the small molecule, GDC-0834, a selective reversible ATP-competitive, Bruton's tyrosine kinase (BTK) inhibitor. A single dose clinical study was designed to determine the compound's pharmacokinetic liability and was preceded by a single dose preclinical toxicology study. The compound was deemed a high-risk candidate for achieving adequate exposure in the clinic due to the high rate of amide hydrolysis found in in vitro human liver fractions; however, the enzyme involved in this hydrolysis was not known (Liu et al., 2011). Despite the potential ADME liabilities, the molecule was advanced to the clinic for three main reasons: (1) the limited experience and lack of understanding in the translatability of in vitro CL_{int} due to hydrolysis to in vivo clearance, (2) the limited amide hydrolysis observed in preclinical species, and (3) the high plasma protein binding (>99%) that could potentially protect the compound from rapid hydrolysis. GDC-0834 failed in the exploratory clinical studies with limited exposure in circulation (<1 ng/ml) after oral administration to healthy volunteers and only an inactive metabolite, M1, was detected in circulation (**Fig. 1**). Although this compound failed in the clinic, the findings allowed the project team to concentrate their efforts on removing this liability in subsequent compounds and advance candidates with optimized pharmacokinetic properties (Young et al., 2016).

The discovery that AO is the enzyme responsible for the amide hydrolysis of GDC-0834 came several years following the clinical study. At the time of the clinical study, from a preclinical, drug discovery point of view, it was sufficient to measure the rates of metabolism in hepatocytes or liver cytosol and ultimately remove this metabolic liability without knowing the specific enzyme(s) involved (Young et al., 2016). Selective chemical

inhibitors and recombinant enzymes are commonly used for the purpose of reaction phenotyping for a handful of enzymes, mainly P450, FMO, UGT, and AO. Although AO is on this list, at the time it was not considered to be involved in the hydrolysis of GDC-0834. Since then, we have been working on developing tools to characterize a variety of drug-metabolizing enzymes by complementing separation techniques with proteomic approaches (see below). As we continued to develop protocols for the characterization of these enzymes, identification of the enzyme(s) involved in the metabolism of GDC-0834 became particularly interesting, in part because the phenotyping of hydrolytic enzymes is quite challenging due to the diversity of enzymes.

The amide hydrolysis of GDC-0834 was originally observed in human liver microsomes in the presence and absence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), but following further investigations, the source of hydrolysis in liver microsomes was determined to be due to the contamination of cytosolic enzymes. We, therefore, focused on developing the tools to identify the cytosolic enzyme involved in this reaction. One advantage of identifying cytosolic enzymes is that the separation of the soluble enzymes is possible while retaining enzymatic activity, as opposed to microsomal enzymes that are membrane-bound. A size-exclusion technique was deployed using phosphate-buffered saline, pH 7.4, as the mobile phase, and the activity for each fraction was determined. For fractions that contained measurable amide hydrolytic activity, samples were loaded on a SDS-PAGE and partially separated and subjected to in-gel trypsin digestion. The peptides were analyzed by various high-resolution mass spectrometry techniques and by comparison of the identified peptide sequence to human proteins. Finally, Pearson correlations were utilized for each protein using the spectral count data relative to the metabolic activity observed across the series of fractions. This analytical technique led to several potential hits with AO being one of the best matches with respect to activity profile and peptide spectral data. Armed with this knowledge, incubations with chemical inhibitors were used to confirm the role of this enzyme. AO inhibitors such as β-estradiol, 2,6-dichlorophenolindophenol (DCPIP), menadione, and raloxifene showed potent inhibition of GDC-0834 hydrolysis with single digit micromolar IC_{50} values. The GDC-0834 inhibition potential against AO-mediated selective substrates was also examined. Six commonly used AO

substrates were used, and the IC_{50} range of GDC-0834 was determined to be 0.86–1.87 μM. Interestingly, carboxylesterase (CES) was also among the potential proteins identified by the proteomic technique. In inhibition studies, the CES inhibitors, bis(p-nitrophenyl)phosphate (BNPP) and loperamide (a mixed inhibitor of both AO and CES), were also able to completely inhibit the amide hydrolysis reaction of GDC-0834 in the micromolar range. The role of CES was further investigated in other species. In dog liver cytosol, in which GDC-0834 was also hydrolyzed, only CES inhibitors, and not AO inhibitors, were capable of inhibiting this reaction. This is consistent with the lack of AO activity in dog liver. Human plasma, which has low levels of CES and no AO (Sharma et al., 2011), also had a limited enzymatic activity for hydrolyzing GDC-0834, and only CES inhibitors were able to prevent the reaction from proceeding. Attempts to separate AO and CES in human liver cytosol were unsuccessful. These results suggest a stark contrast between CES activity in human plasma and human liver cytosol. The difference in these activities might be due to the presence of both AO and CES in liver subcellular fractions, resulting in the dual contribution of these enzymes to GDC-0834 hydrolysis in liver cytosol. Although both AO and CES were identified by mass spectrometry-proteomics analysis of the enzymatically active fractions and the relative contributions of AO compared to CES in the amide hydrolysis of GDC-0834 remains unanswered, the proteomic analysis of the fractions and the enzyme activity profile of AO in the cytosolic fractions was a better correlation than that of CES. The findings from these studies provide convincing evidence for the role that AO plays in the amide hydrolysis of GDC-0834.

A mechanism of AO-mediated hydrolysis was postulated. AO is a homodimer with 150 kDa subunits and is composed of three distinct domains: a flavin adenine dinucleotide (FAD) binding domain, two iron-thiol containing cluster domains, and a molybdenum pyranopterin cofactor (MoCo). The Mo^{VI} center is considered essential for enzymatic catalysis. Even though this enzyme is named for the oxidation of an aldehyde to a carboxylic acid, its contribution to the oxidation of aromatic aza-heterocycles is considered to be more important in drug metabolism. In a typical proposed reaction mechanism, a hydroxymolybdenum group with Mo^{VI} is

deprotonated by Glu1270 (Dastmalchi and Hamzeh-Mivehrod, 2005) and attacks a sp^2 carbon center next to a heteroatom (**Fig. 2**, Step 1A). With assistance from the heteroatom, the hydride on the same carbon atom is heterolytically cleaved and forms a new thiol bond. The thiol consequently donates two electrons to the Mo center, resulting in a reduction to Mo^{IV} (Step 2A). A water molecule then reacts with the carbon center to form a tetrahedral intermediate (Steps 3A and 4A). This intermediate then collapses to separate the oxidized product from the Mo^{IV} center (Step 5A). The Mo^{IV} donates two electrons through MoCo to the iron-thiol clusters and finally to FAD, leading to the reformation of Mo^{VI} and to the reduction of oxygen gas to various reactive oxygen species (ROS; Step 6A).

Although this mechanism is a reasonable for oxidation, it does not completely explain the amide hydrolysis observed for GDC-0834. In the case of an amide bond, the sp^2 carbon atom attached to two heteroatoms was postulated to be attacked. In order to have the electron density necessary to attack the electrophilic carbon center of the amide, the oxidation state of Mo is postulated to start at Mo^{IV}, rather than the more typically considered Mo^{VI}. The proximity of the amide bond to MoCo was confirmed by a docking experiment that shows that the amide bond is exposed to the hydroxyl-molybdenum without interference from the rest of the molecule. The reaction mechanism begins with an attack of the hydroxyl group to the sp^2 carbon center (Step 1B), resulting in a tetrahedral intermediate that collapses to free the amine (M1 metabolite of GDC-0834; Step 2B). The newly formed ester center is attacked by a water molecule (Step 3B) that frees the enzyme at the same oxidation state as the starting material (i.e. Mo^{IV}). This mechanism is similar to that of the oxidation reaction in **Fig. 2A** except that in the oxidation, the Mo^{IV} is postulated to be converted back to Mo^{VI} via formation of ROS at the FAD center.

In conclusion, until recently, the main role of AO in drug metabolism was thought to involve the oxidation of aromatic aza-heterocycles and a few reduction reactions. In light of these new findings, the involvement of AO should also be considered in the mediation of amide hydrolysis.

Flavin-containing monooxygenase (FMO)

The FMO family of membrane-bound enzymes is associated with the endoplasmic reticulum and is active in microsomal subcellular fractions. Similar to P450s, FMOs require NADPH as a co-factor and typically catalyze the addition of an oxygen atom to substrates that contains a nucleophilic heteroatom with a lone pair of electrons. This type of oxidation mechanism is well known for FMOs (Cashman, 2008); however, reports on carbon oxidation by FMOs are less common. Here we examine two FMO-mediated carbon oxidation reactions in detail. Several P450 reactions are known to mimic FMO reactions, and therefore various techniques have been developed to differentiate between the contributions of each of these enzymes. These techniques include the use of FMO inhibitors (i.e., methimazole), recombinant enzymes, heat treatment of microsomes (50 □C inactivates FMO), and/or varying the pH (pH of 8.5–9.5 is optimum for FMOs) (Cashman, 2005). However, in the absence of a shift in FMO activity by use of these techniques, the contribution of FMOs at times cannot be distinguished from that of P450s. Due to the challenges associated with differentiating oxidations between P450 and non-P450 enzymes, it is not surprising that initially we attributed the oxidative defluorination reaction to P450s. Upon further studies using recombinant enzymes and selective P450 or FMO inhibitors, 1-aminobenzotriazole (ABT) and methimazole, respectively, we determined that this reaction could actually be carried out by FMOs. Since carbon oxidation via FMOs is uncommon, we decided to investigate this reaction using a simpler probe, 4-fluoro-*N*-methylaniline (**1**, **Fig. 3**), in order to gain insight into the reaction mechanism. Boersma et al. (1993) first proposed that **1** is converted to 4-(methylamino)phenol (**2**) via FMOs by using rat purified FMO and ^{19}F-NMR. **2** was further characterized using FMO inhibitors in liver microsomes. Investigations were undertaken to elucidate this mechanism using trapping studies in addition to labeled water and oxygen gas to determine the source of the oxygen atom in **2** (Driscoll et al., 2010). The origin of the incorporated oxygen in **2** was determined to be from O_2, as is expected from an FMO-type reaction. In the proposed mechanism, the lone-pair of electrons from the aniline nitrogen contribute to formation of an imine, which ultimately results in a new bond between the carbon atom at the 4 position of **1** and the distal oxygen of FAD-OOH (**Fig. 3**). To

further support the proposed mechanism, ab initio calculations were performed and confirmed the donation of electrons from the *N*-methylaniline nitrogen. The intermediate then collapses, resulting in the release of HF and eventual formation of **2**. If this mechanism were correct, a quinoneimine intermediate would need to form. To test this hypothesis, incubations with 4-fluoro-*N*-methylaniline were conducted in rat liver microsomes fortified with NADPH and the trapping agent, glutathione (GSH). No detectable levels of GSH conjugates were observed. Interestingly, when *N*-acetylcysteine (NAC) was used as a trapping agent instead of GSH, NAC-conjugates were detected. We attribute this difference in the formation of conjugates to the smaller size of NAC that allows the compound to access the active site of FMO, while the larger GSH molecule could not. The formation of NAC-conjugates indicates the formation of a quinoneimine intermediate.

Another important process involving carbon oxidation by FMO is the Baeyer-Villiger (BV) reaction. Several reported examples of the BV reaction include salicylaldehyde (Chen et al., 1995) and 4-piperidinone-containing compounds such as E7016 (Lai et al., 2011, **Fig. 4B**) and MRX-1 (Meng et al., 2015, **Fig. 4C**). In the reaction with 4-piperidinone-containing compounds, an oxygen atom is inserted alpha to the carbonyl moiety, resulting in the formation of an ester. The ester is then hydrolyzed to a ring opened alcohol and carboxylic acid. E7016 and MRX-1 illustrate specificity for FMO5 since the other isoforms did not lead to formation of a product. FMO5 is thought to have a deeper binding pocket than either FMO1 or FMO3 on the basis of data collected from long chain hydrocarbons with terminal tertiary amines (Cashman, 2008). E7016 and MRX-1 could possibly be employed as specific probes for FMO5 activity.

FMO5 typically does not get a lot of attention due to previous thoughts that it had very narrow substrate specificity and low activity to typical FMO substrates (Cashman & Zhang, 2006). The enzyme is thought to have a deeper binding pocket than either FMO1 or FMO3 on the basis of data collected from long chain hydrocarbons with terminal tertiary amines (Cashman, 2008). Recent effort on the catalytic properties of FMO5 as a Bayer-Villiger mono-oxygenase was published (Fiorentini et al., 2016). This work took a set of cyclic and non-cyclic ketones and aldehydes and performed in vitro experiments with hFMO5. The results show that this

enzyme can catalyze Bayer-Villiger reactions on a fairly wide set of substrates containing ketones and aldehydes. FMO continues to be one of the oxidative enzymes that does not receive much exposure in the literature. However, it is an important enzyme that may contribute to both heteroatom and carbon atom oxidation. Being able to dissect the relative contribution of FMOs compared to P450s may allow for different strategies to be deployed for better understanding and optimization during the drug discovery stage.

Gamma-Glutamyl Transpeptidase (GGT)

GGT is a membrane-bound glycoprotein consisting of two subunits of 51 and 22 kDa (Tate and Meister, 1976) and is mainly present on the luminal surface of the proximal tubules in the kidney as well as in bile ducts of the liver (Tate and Meister, 1981). The main function of GGT is to hydrolyze GSH conjugates or GSH at the γ-linkage between the γ-carboxyl group of glutamate and the α-amino group of cysteine in GSH, leaving the cysteinyl-glycine peptide susceptible to additional cleavage by aminopeptidases so that GSH can be hydrolyzed to amino acids for renal reabsorption (Pompella et al., 2006). Studies with the GGT suicide inhibitor, L-gamma-glutamyl-(*O*-carboxy)phenyl-hydrazine, resulted in elevated GSH levels (3,000-fold) in rat urine, which provided direct evidence to support this role of GGT (Griffith and Meister, 1979). Hydrolysis by GGT is considered the first step in the conversion of GSH conjugates of xenobiotics to cysteinyl-glycine that ultimately generates mercapturic acids that are eliminated either in urine or bile (Lohr et al., 1998; **Fig. 5A**).

The role of GGT in drug discovery is highlighted in several cases in which GSH conjugates are further metabolized to form a free thiol by β-lyase that, in some cases, are nephrotoxic metabolites. A couple examples of this include cisplatin and efavirenz (Dekant, 2001). In the case of cisplatin, the dose limiting toxicity is nephrotoxicity in cancer patients (Ries and Klastersky, 1986). The highly expressed GGT at the luminal surface of proximal tubule cells hydrolyze the extracellular cisplatin-GSH complex in glomerular filtrate to produce a cisplatin-cysteinyl-glycine intermediate that is further hydrolyzed by another cell surface membrane enzyme, amino-dipeptidase. The resulting cysteine-cisplatin conjugate is then taken up by tubule cells and converted to a highly toxic and reactive thiol by cysteine *S*-conjugate β-lyase (the intermediate in Figure 5C upper right corner) (Zhang and Hanigan, 2003; **Fig. 5B**). In agreement with this mechanism, no nephrotoxicity was observed in GGT knockout mice or pre-treatment with the GGT inhibitor acivicin in mice (Hanigan et al., 1994; 2001).

Another example of GGT-mediated nephrotoxicity is that of efavirenz, an HIV reverse transcriptase inhibitor. Efavirenz is interesting in that it exhibits rat-specific nephrotoxicity that is not observed in either monkey or human (Mutlib et al., 1999). The proximal toxic metabolite is formed only in rats through the formation and

subsequent processing of the GSH conjugate to a sulfate metabolite, which was postulated to be responsible for the species-specific renal toxicity in rats (Mutlib et al., 2000). Metabolism studies showed that rodent-specific glutathione *S*-transferase (GST) formed **3** (**Fig. 5C**) in rats and mice but not in monkey or human. Intermediate **3** is further hydrolyzed by GGT to the cysteinylglycine conjugate in the kidneys of rats, and it is this metabolite that leads to the ultimate nephrotoxic metabolite in the kidney (Mutlib et al., 1999). This finding clearly demonstrates the value of metabolic comparison studies in animal species to advance a drug discovery program. In summary, GGT is the first upstream enzyme involved in the hydrolysis of GSH conjugates. The products of this hydrolysis, mainly cysteinylglycine conjugates, do not play a role in toxicity in the kidney; however, several cases exist in which these conjugates lead to nephrotoxicity due to formation of proximate toxic metabolites.

The drug-induced nephrotoxicity have been reported for a number of halogenated compounds such as trichloroethene and hexachloro-1,3-butadiene. Similar mechanisms have been proposed to involve GGT-mediated hydrolysis of glutathione adducts and subsequent processing to generate reactive and toxic thiol metabolites (Dekant, 2001). Formation of glutathione adducts is considered as an important clearance pathway for recently discovered targeted protein covalent inactivators (Singh et al., 2011).

Cathepsin B

Cathepsin B is a lysosomal enzyme whose enzymatic activity has been targeted for hydrolysis of prodrugs and antibody-drug conjugates (ADCs) to introduce the active drug to specific tumors. Cathepsin B is both an endopeptidase and a carboxypeptidase (Vasiljeva et al., 2007), and its physiological function is in the turnover of proteins and in maintaining the normal metabolism of cells. Concentrations of cathepsin B can reach up to 1 mM in human tumors (Mohamed and Sloane, 2006) and, as is expected from lysosomal enzymes, its catalytic activity is optimal under acidic conditions (Conus and Simon, 2010). The enzyme is composed of a dimer of disulfide-linked heavy and light chains (~30 kDa) and belongs to the superfamily of papain-like cysteine proteases.

The ADC is a relatively old concept that has come to fruition with the recent approval of two ADCs, brentuximab vedotin (Adcetris®) and ado-trastuzumab emtansine (Kadcyla®, T-DM1) and the more than 40 ADCs in clinical trials (Chari et al., 2014). Some ADCs are designed such that the linker is a substrate of cathepsin B and is rapidly cleaved in the lysosomes of the tumor (**Fig. 6**). One such linker is valine-citrulline (Val-Cit), which is hydrolyzed by cathepsin B. Cathepsin B is also targeted in prodrug approaches. Paclitaxel linked through the tetrapeptide Gly-Phe-Leu-Gly has been used in the design of prodrugs that target cathepsin B in tumors (Satsangi et al., 2014; **Fig. 6A**) This prodrug showed a higher cytotoxicity in cell lines with moderate to high expression of cathepsin B than in those with low expression. The conjugate also showed a higher tumor size reduction than paclitaxel in xenograft models. In developing therapy to treat bone cancers, doxorubicin was conjugated to bisphosphonates that was highly accumulated in bone and bone metastases to enhance its effectiveness (Zhong et al., 2013; **Fig. 6B**). The bisphosphonate, bone-seeking agents demonstrate an uptake in bone of up to 20–80% of the administered doses, and, more importantly, the uptake in bone metastases might be 10–20-fold higher than in healthy bone tissue (Hirabayashi et al., 2001). Such a design ensures an effective release of doxorubicin at the site of action. Shao et al. (2012) also used a novel prodrug, acetyl-Phe-Lys-PABC-doxorubicin, to demonstrate the utility of the cathepsin B cleavable linker (**Fig. 6C**). para-Aminobenzyl (PAB) undergoes

self-immolation to release to cytotoxic drug. This prodrug shows a low dose-dependent inhibitory effect on the growth of the gastric cancer cell line SGC-7901 and significantly lowered bone marrow, kidney, liver and heart toxicities in mice, thus making this an effective targeting drug to treat gastric cancer. The linking of doxorubicin to *N*-(2-hydroxypropyl)methacrylamide copolymers (FCE 28068) via a cathepsin B-cleavable tetrapeptide spacer (Duncan et al., 1983) enhanced permeability and the retention effect. FCE 28068 was also linked to galactosamine, which binds to the hepatic asialoglycoprotein receptor to achieve liver-specific doxorubicin delivery (Seymour et al., 2002). Val-Cit is linked to para-aminobenzyl (PAB) to self-immolate to release to cytotoxic drug into the tumor. Such a design is used in brentuximab vedotin (Adcetris®) and is approved to treat refractory Hodgkin's lymphoma and anaplastic large cell lymphoma (Chari et al., 2014; **Fig. 6D**). After internalization of the antigen-receptor complex, cytotoxic drugs such as calicheamycin, maytansine, duocarmycin, auristatin, and irinotecan are released intracellularly after linker cleavage (Carter and Senter, 2013). Cathepsin B is a targeted enzyme used to release an active drug at the site of action. This approach has been used effectively in the ADC field as well as in prodrug design of small molecules and has opened doors to new therapeutic targets. This is especially true in oncology. The result is a maximization of drug effect at the site of action in tumors by taking advantage of the elevated expression of cathepsin B. Note that cathepsin B cleavable peptidomimetics have also been shown tp be cleaved by other protease enzymes such as cathepsin C, F, H, K, and L (Mohamed and Sloane, 2006).

ADP-ribosyltransferase (ART)

Ribose conjugation is another uncommon metabolic pathway that contributes to the formation of polar metabolites, similarly to the more well-known carbohydrate conjugates, glucuronide and glucose. The enzyme responsible for ribose conjugation is ART (Le et al., 2013). The ribose conjugation modifies azo-heterocycles or hydroxyl-containing moieties (Kulkarni and Hodgson, 1980, **Fig. 7**). One of the members of the ART superfamily, NAD^+ hydrolase, also known as glycohydrolase, nucleosidase or NADase (EC 3.2.2.5), is the enzyme responsible for the hydrolysis of NAD^+ to form ADP-ribose plus nicotinamide and is also involved in ribose conjugation. The reaction is proposed to proceed via either a direct transfer (pathway A, **Fig. 8**) or a two-step process (pathway B) specifically for NADase: first, a highly reactive oxonium ion species is generated, followed by S_N1 nucleophilic attack of a nitrogen or oxygen (Oppenheimer, 1994) with nicotinamide acting as a leaving group. Examples of substrates are rifampin, imatinib (Miao et al., 2012), otenabant, natural toxin 4-ipomeanol (Chen et al., 2006), nitrosamines, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) (Peterson et al., 1994) and 6-(1H-pyrazol-4-yl)-*N*-(1-(thiazol-4-ylmethyl)-1H-pyrazol-4-yl)-1H-indazole-3-carboxamide (**4**). Rifampin ribose conjugation has been reported to lead to the rapid deactivation of its antibiotic activities towards bacteria (Baysarowich et al., 2008).

Our interest in this metabolic pathway started with a series of interleukin-2-inducible T-cell kinase (ITK) inhibitors that had demonstrated high clearance in rat hepatocyte metabolic stability assays. One such compound was **4**, which exclusively formed ribose conjugates in rodent hepatocytes (Le et al., 2013). In cytosol, only the adenine dinucleotide ribose (ADR) conjugate was detected with NAD^+ as the cofactor. Liver cytosol only fortified with $NADP^+$ failed to generate the corresponding conjugate in any significant amount. In microsomes, however, either NAD^+ or $NADP^+$ served as an efficient ribose source for **4**. Taken together, this suggests that the enzyme responsible for ribosylation is different in these two matrices, with the enzyme in microsomes being membrane-bound. Interestingly, nicotinamide mononucleotide (NMN) and reduced form of

NAD(P) performed efficiently well in our subcellular fractions, suggesting that the former cofactor and the reduced form are also able to supply ribose. In hepatocytes, ribose conjugates were detected instead of the full length adenine dinucleotide phosphate ribose (AD(P)R) conjugates. After addition of a phosphatase inhibitor cocktail, the ADR conjugate was detected in hepatocytes, which suggests that plasma membrane phosphatase in hepatocytes may play an important role in hydrolyzing the phosphate bond and releasing the ribose conjugate as the final product. In silico estimation of binding for **4** were very useful to determine the specific binding of the **4** in the active site of ART. From a structural point of view, **4** contains tetrameric aromatic rings, but the terminal pyrazole was identified as the likely active site for ART. Interestingly, replacing the distal thiazole group in **4** to a phenylpropyl group (or (*S*)-*N*-(1-(1-phenylpropyl)-1H-pyrazol-4-yl)-6-(1*H*-pyrazol-4-yl)-1*H*-indazole-3-carboxamide), **5**, removed the key interactions and eliminated the ribose conjugation liability.

ADP-ribosylation in proteins has been shown to be responsible for many post-translational modifications, such as in apoptosis (Bricker et al., 2005), cell signaling (Ziegler et al., 1997), DNA repair (Althaus et al., 1982) and gene regulation (Ryu et al., 2015). Such modification often results in the inactivation of target proteins (Haag and Koch-Nolte, 1997). Conversely, NAD^+ hydrolase is responsible for the reactivation of modified proteins by hydrolyzing the conjugate.

In summary, ADP-ribosylation is highly species specific and is mainly observed in rodents (i.e., imatinib, ipomeanol, NNK/NNAL and **4**) but not in higher species such as dog, monkey and human. ADP conjugate, specifically **4**, is subject to rapid phosphatase hydrolysis to form ribose conjugates. Finally, the biological importance of this biotransformation pathway requires more research.

Conclusion

Metabolism is considered the most important pathway for the elimination of drugs from the body, and the significant roles of P450 and UGT enzymes in this process are well established. Here we present a variety of examples that demonstrate the contributions of other Phase I and Phase II enzymes to the metabolism and elimination of drugs. These enzymes are responsible for the high clearance of the human-specific amide hydrolysis of GDC-0834 by AO and the rodent-specific ribose conjugation by ART. GGT is an enzyme that is involved in the initial hydrolysis of glutathione metabolites that can lead to nephrotoxicity, as in the case of human-specific cisplatin or rodent-specific efavirenz. Hydrolysis by cathepsin B has been targeted to release potent cytotoxins, as in the case of several ADCs. Finally, carbon oxidation, which is usually attributed to P450 enzymes, can also be a result of FMO-mediated metabolism. All these examples show that a wide array of drug metabolizing enzymes should be examined during the design and evaluation of molecules in drug discovery. That is, understanding the nature of these enzymes and their mechanisms will allow rational design of better prodrugs or drugs with improved safety or PK profiles. Species unique metabolism or bioactivation can also be better evaluated based on the examples presented in this paper.

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Participated in research design:

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Legends for Figures:

Fig. 1. Enzymatic hydrolysis of GDC-0834 in human liver cytosol to form M1 and M2.

Fig. 2. (A) The mechanism of aldehyde or aromatic aza-heterocyclic oxidation by aldehyde oxidase (AO). When X is an oxygen atom, R2 is an electron lone pair and when X is a nitrogen atom, R1 and R2 are substituents. (B) The proposed mechanism for amide hydrolysis by AO.

Fig. 3. Mechanism of oxidative defluorination of 4-fluoro-*N*-methylaniline (**1**) by rat FMO and formation of 4-(methylamino)phenol (**2**). NAC is *N*-acetylcysteine.

Fig. 4. (A) Overall mechanism of the Baeyer-Villiger reaction by FMO and (B) and (C) are specific examples of such reactions catalyzed by FMO5.

Fig. 5. (A) Glutathione (GSH) conjugates are first hydrolyzed by gamma-glutamyl transpeptidase (GGT) to cysteinylglycine conjugates, which are subsequently further hydrolyzed by amino dipeptidase to cysteine conjugates. These conjugates can be either conjugated by *N*-acetyl transferase to form mercapturic acids or further cleaved by β-lyase to form possible thiol conjugates (Drug-S^-). Cisplatin (B) and efavirenz (C) are two examples in which these reactions are observed. GST is glutathione *S*-transferase and SULT is sulfotransferase.

Fig. 6. Cathepsin B-cleavable linkers and associated with ADC and prodrugs. The arrows show the site of hydrolysis by this enzyme system. (A) is dendrimer-Gly-Phe-Leu-Gly-paclitaxel, (B) is bisphosphonate-Val-Ala-PABC-doxorubicin, (C) is acetyl-Phe-Lys-PABC-doxorubicin, and (D) is brentuximab vedotin.

Fig. 7. Examples of small molecule ADP-ribosylation. The arrow shows the site of ribose conjugation. **4** is 6-(1*H*-pyrazol-4-yl)-*N*-(1-(thiazol-4-ylmethyl)-1*H*-pyrazol-4-yl)-1*H*-indazole-3-carboxamide, **5** is (*S*)-*N*-(1-(1-phenylpropyl)-1H-pyrazol-4-yl)-6-(1*H*-pyrazol-4-yl)-1*H*-indazole-3-carboxamide, NNK is

4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, NNAL is
4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol.

Fig. 8. Enzymes and cofactors responsible for ribose conjugation of nitrogen and oxygen-containing xenobiotics: The first step is the transfer of ADP from either NAD/P or NMN to the accepting xenobiotic followed by phosphatase hydrolysis (proposed pathway A or B; pathway A is direct transfer of cofactor to the acceptor nucleophile; pathway B was proposed by Oppenheimer (1994) for NADase in a two-step process. ADP-ribose conjugates have been reported in rodent liver microsomes, cytosol and pig brain homogenate, whereas ribose conjugates have been reported in rodent hepatocytes and in vivo excreta. NAD = nicotinamide adenine dinucleotide and its reduced form, NADP = nicotinamide adenine dinucleotide phosphate and its reduced form, NMN = nicotinamide mononucleotide.

Fig. 1.

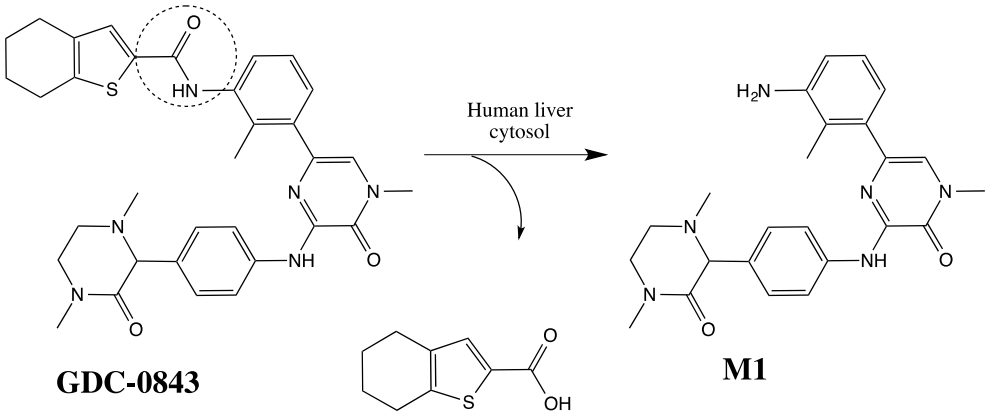


Fig. 2.

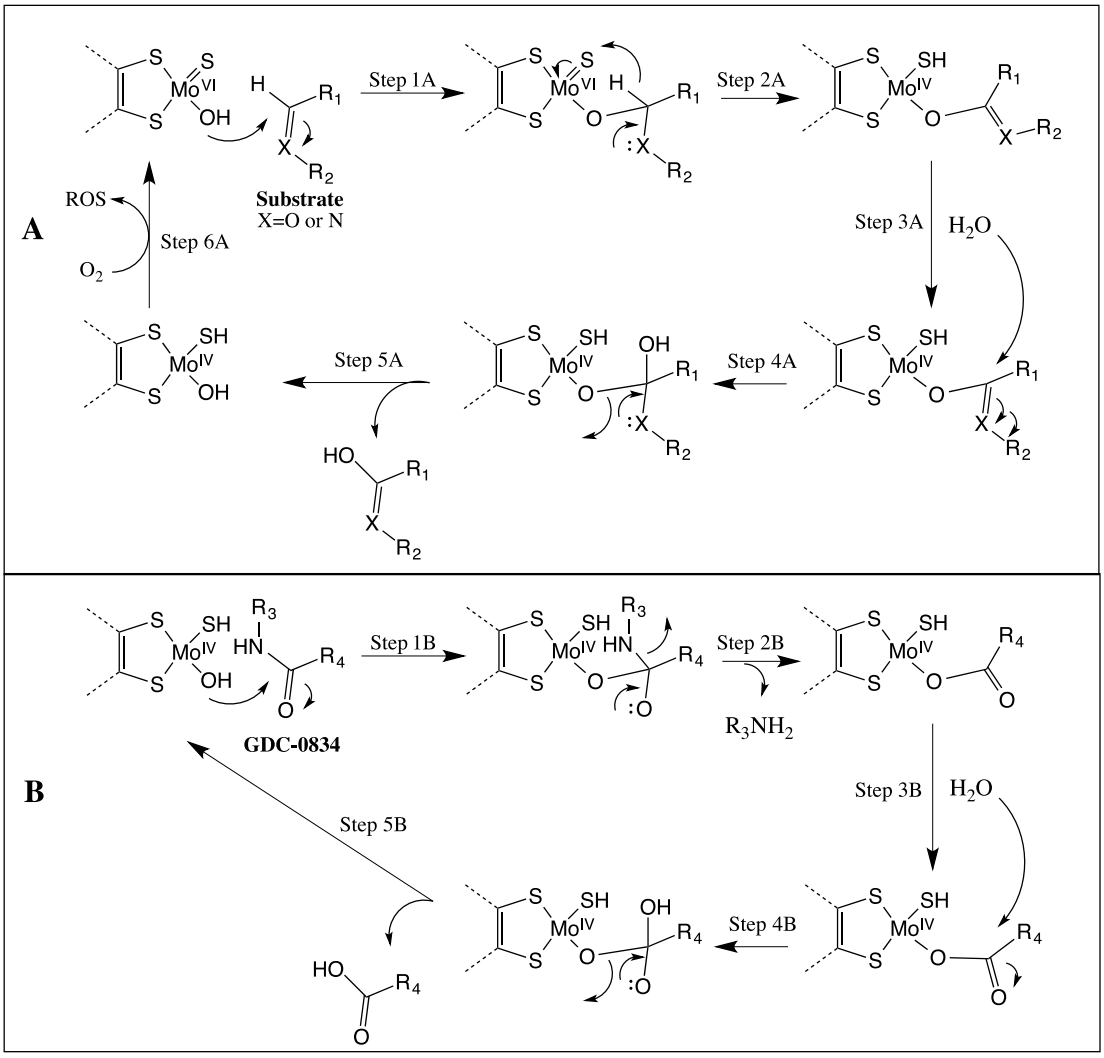

A
Step 1A
Step 2A
Step 3A
H_2O
Step 4A
Step 5A
Step 6A
ROS
O_2
Substrate
X=O or N
B
Step 1B
Step 2B
R_3NH_2
Step 3B
H_2O
Step 4B
Step 5B
GDC-0834

Fig. 3.

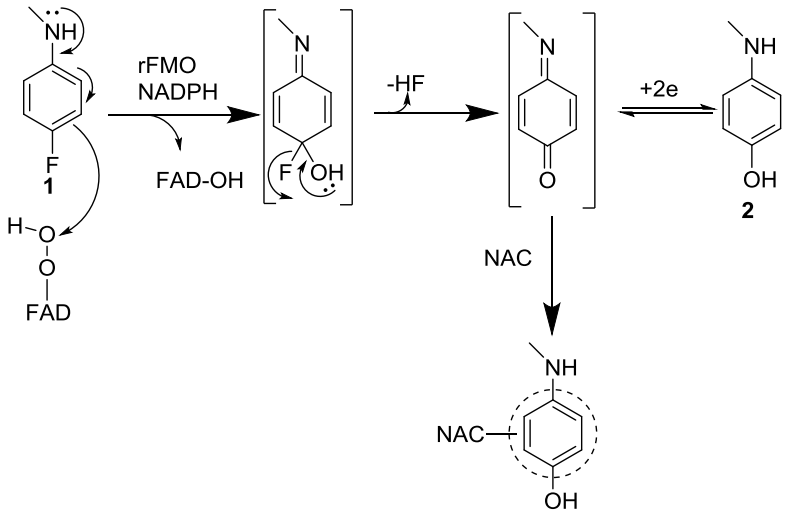


Fig. 4.

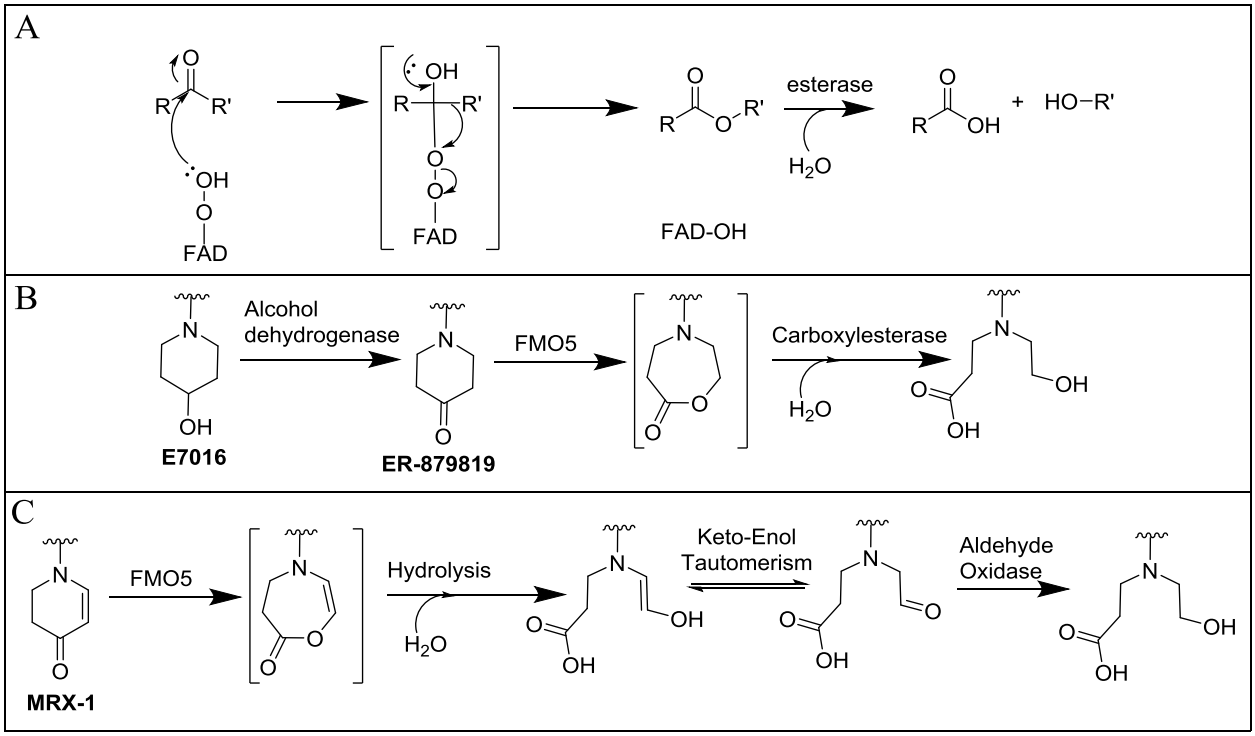

A
esterase
FAD-OH
B
Alcohol
dehydrogenase
FMO5
Carboxylesterase
E7016
ER-879819
C
FMO5
Hydrolysis
Keto-Enol
Tautomerism
Aldehyde
Oxidase
MRX-1

Fig. 5.

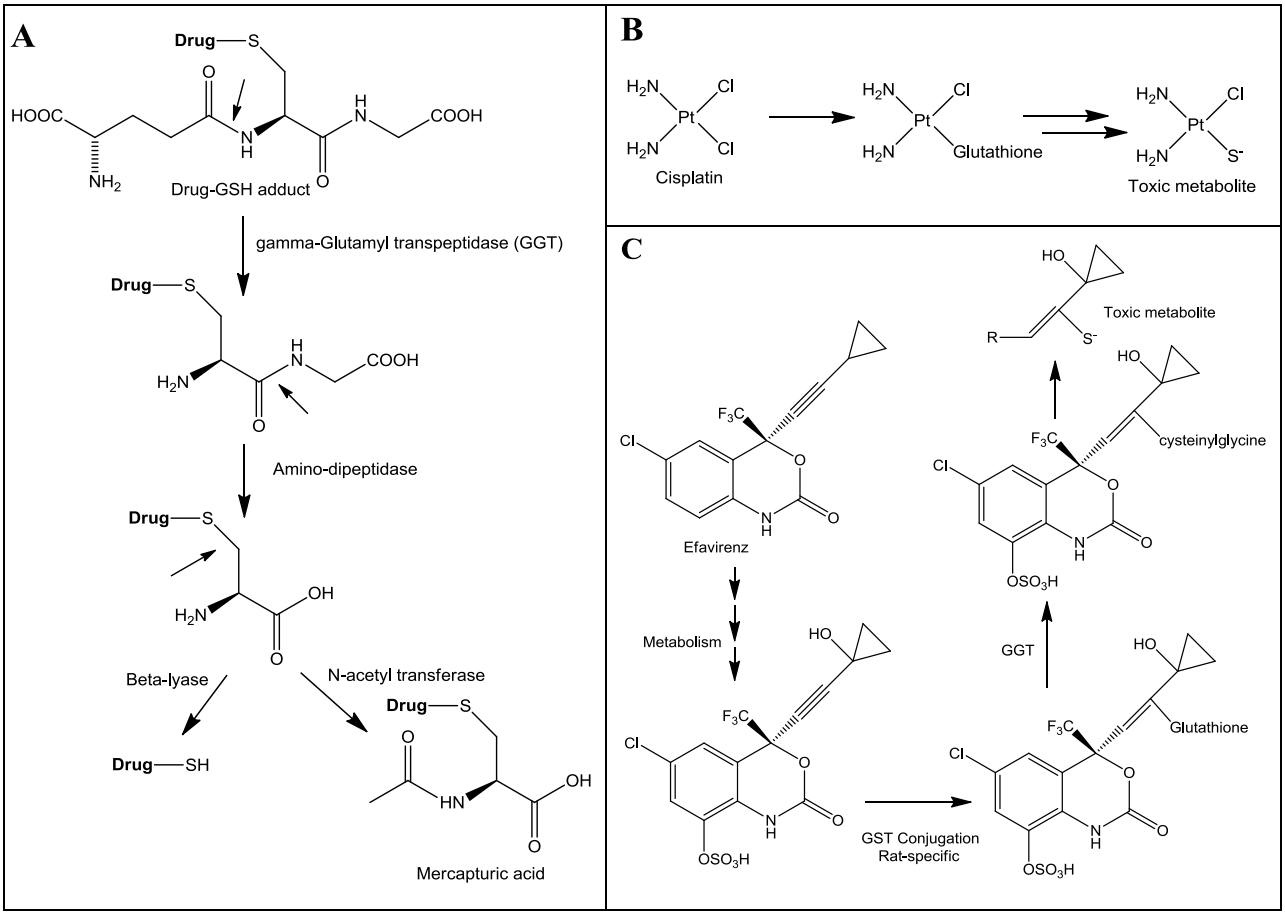

A
Drug—S
HOOC
COOH
NH2
Drug-GSH adduct
gamma-Glutamyl transpeptidase (GGT)
Amino-dipeptidase
Beta-lyase
N-acetyl transferase
Drug—SH
Mercapturic acid
B
Cisplatin
Glutathione
Toxic metabolite
C
Efavirenz
Metabolism
GST Conjugation
Rat-specific
GGT
Toxic metabolite
cysteinylglycine
Glutathione

Fig 6.

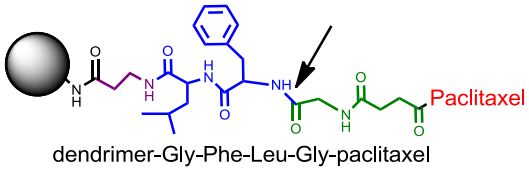
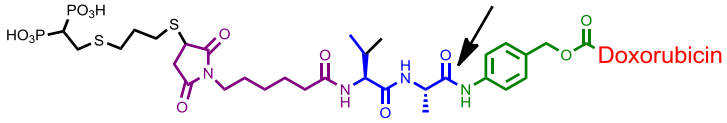
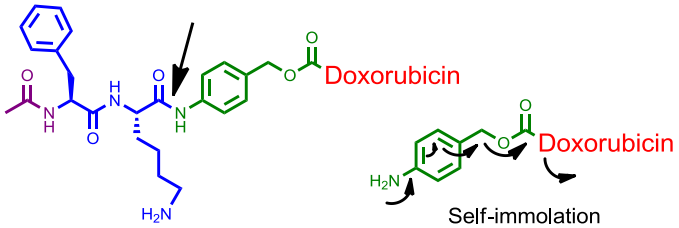
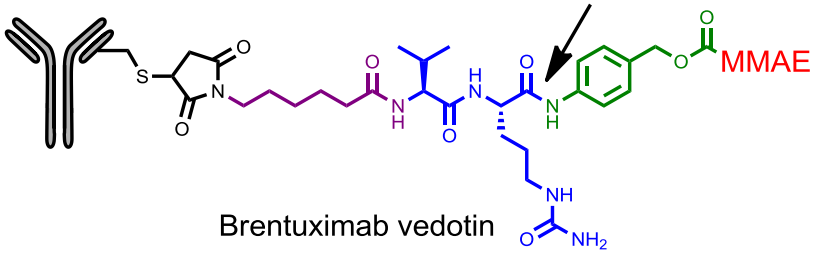
A	 dendrimer-Gly-Phe-Leu-Gly-paclitaxel
B	 Bisphosphonate-Val-Ala-PABC-doxorubicin
C	 Ac-Phe-Lys-PABC-doxorubicin
D	 Brentuximab vedotin

Fig. 7.

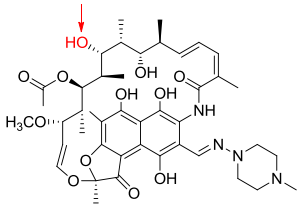
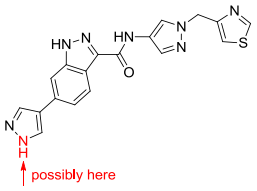
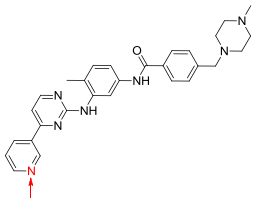
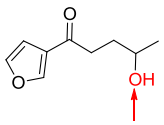
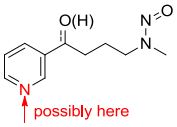
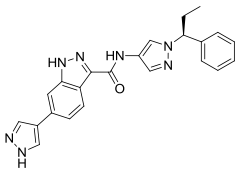
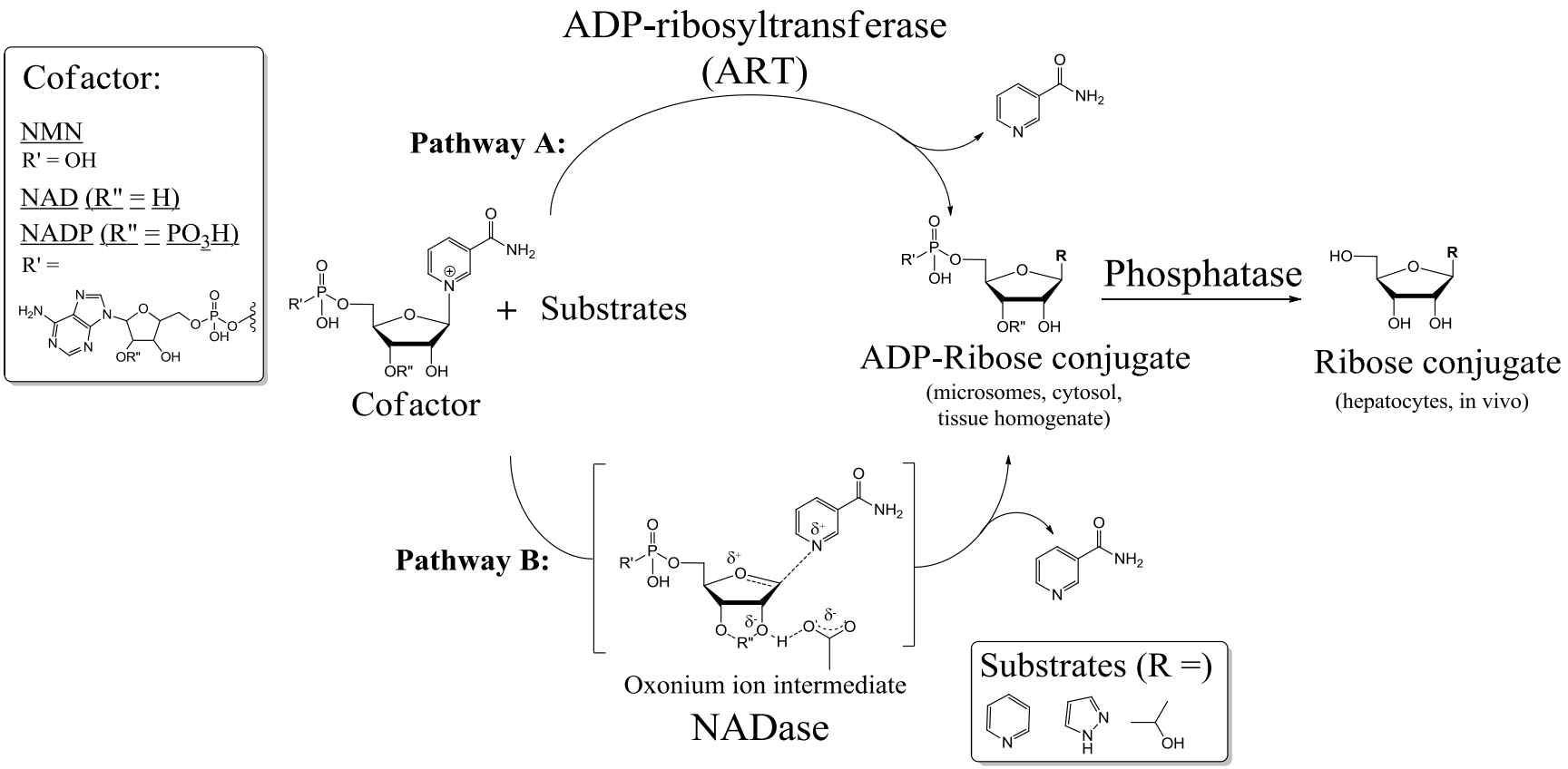
Rifampin	**4**	Imatinib	4-Ipomeanol	NNK/NNAL
				
	5 (not an ART substrate)			
				

Fig. 8.


Cofactor:
NMN
R' = OH
NAD (R" = H)
NADP (R" = PO3H)
R' =
ADP-ribosyltransferase
(ART)
Pathway A:
+ Substrates
Cofactor
ADP-Ribose conjugate
(microsomes, cytosol,
tissue homogenate)
Phosphatase
Ribose conjugate
(hepatocytes, in vivo)
Pathway B:
Oxonium ion intermediate
NADase
Substrates (R =)