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Review Article

Structural perspectives of the CYP3A family and their small molecule modulators in drug metabolism☆

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ABSTRACT

Cytochrome P450 (CYP) enzymes function to catalyze a wide range of reactions, many of which are critically important for drug response. Members of the human cytochrome P450 3A (CYP3A) family are particularly important in drug clearance, and they collectively metabolize more than half of all currently prescribed medications. The ability of these enzymes to bind a large and structurally diverse set of compounds increases the chances of their modulating or facilitating drug metabolism in unfavorable ways. Emerging evidence suggests that individual enzymes in the CYP3A family play discrete and important roles in catalysis and disease progression. Here we review the similarities and differences among CYP3A enzymes with regard to substrate recognition, metabolism, modulation by small molecules, and biological consequence, highlighting some of those with clinical significance. We also present structural perspectives to further characterize the basis of these comparisons.

1. Introduction

The human cytochrome P450 (CYP) 3A family is part of the broader CYP superfamily of heme-containing enzymes. The CYP3A enzymes are critical for metabolizing both endogenous and exogenous compounds, and they have been reported to metabolize more than half of all currently prescribed drugs.[1] Members of the CYP3A family include CYP3A4, CYP3A5, CYP3A7, and CYP3A43. These enzymes catalyze various reactions and have exceptionally broad substrate specificity. Because of their ability to interact with structurally diverse compounds, CYP3A enzymes have a high capacity for modulation to change drug responses. CYP3A4 and CYP3A5 are the best characterized members of the family and are reported to be mostly functionally redundant, but they do exhibit differences in their regulation and messenger RNA (mRNA) expression, as we have reviewed previously.[2] CYP3A4 is considered the most important drug-metabolizing enzyme in the body and is the most abundant isoform in the liver, whereas CYP3A5 is the primary source of extra-hepatic CYP3A. CYP3A7 is primarily expressed in fetal liver.[3,4] CYP3A43 remains poorly characterized but does have clinical significance stemming from its genetic variation.[5,6] CYP3A enzymes display high protein sequence homology (Fig. 1), which is the basis of the functional redundancy assumptions made by many researchers. Although several differences in substrate specificity or catalytic efficiency have been reported within the CYP3A family,[7,8] one of the most striking differences was recently highlighted by evidence implicating CYP3A5 specifically in the progression of pancreatic ductal adenocarcinoma (PDAC).[9] Structural evidence has been obtained for both CYP3A4 and CYP3A5, enabling investigations of the subtle differences between their active sites. In this review, we report some of the differences in the drug metabolism and modulation profiles within the CYP3A family. Clinically relevant consequences of the differences between these enzymes are discussed, and structural perspectives are provided to further the understanding of isoform-selective effects.

2. Interplay between CYP3A and small molecules

2.1. Roles involving endogenous compounds

Most current research involving enzymes of the CYP3A family relates to their modulation by, or interactions with, exogenous

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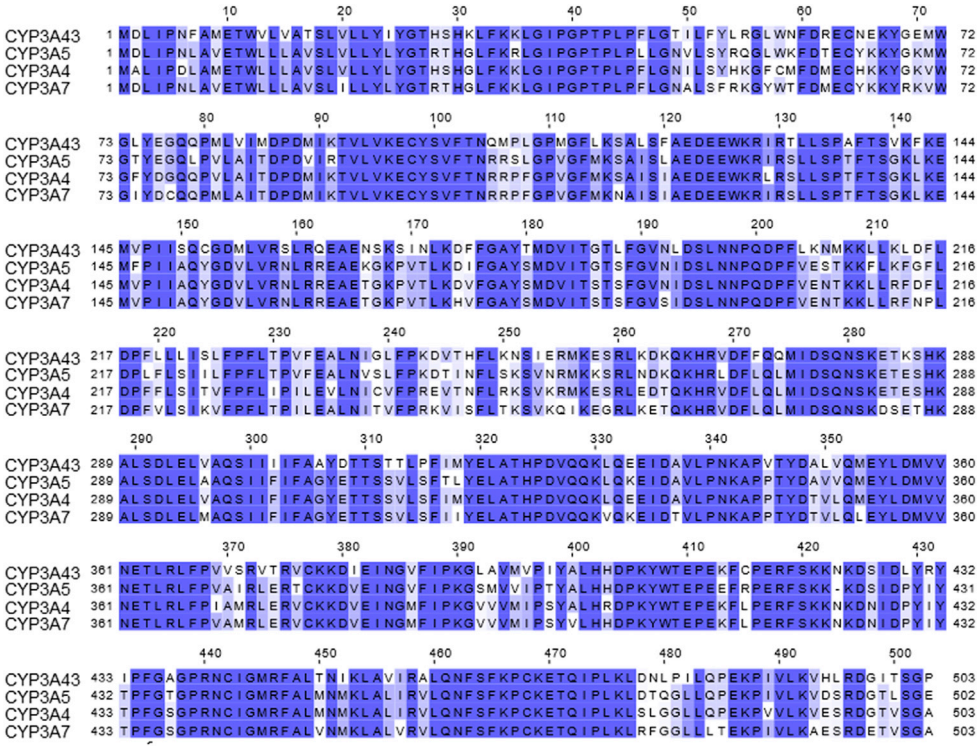

Fig. 1. Protein sequence homology among CYP3A enzymes. Members of the human CYP3A family were aligned and colored by protein sequence homology, with blue indicating a complete match and white indicating a residue deviation. Abbreviation: CYP3A, cytochrome P450 3A.

compounds, primarily those intended for medicinal purposes. However, important and clinically relevant information has been obtained from studies of the endobiotic roles of these enzymes. Although CYP3A enzymes act on a wide range of structurally diverse compounds, most studies of these enzymes in the context of endogenous metabolism have focused on substrates with structural similarities. For example, CYP3A members play an important role in bile acid and steroid metabolism. CYP3A4, CYP3A5, and CYP3A7 selectively catalyze the 1β-hydroxylation of the secondary bile acid deoxycholic acid.[10] The 1β-hydroxydeoxycholic acid (1β-OHDCA) metabolite has been proposed as a urinary biomarker with which to assess potential CYP3A-mediated drug–drug interactions.[10] Cholesterol, another endogenous steroid, also has a metabolite produced by CYP3A4 and CYP3A5, which again demonstrates the clinical relevance of the CYP3A family. Mao *et al.*[11] reported on the suitability of the metabolite 4β-hydroxycholesterol (4β-HC) for studying CYP3A inducers such as the well-established inducer rifampicin.[12] Knowledge of the CYP3A inducibility potential contributed by a given drug is key to understanding the response of that drug. Furthermore, several inducers of CYP3A members act on upstream regulators and have been implicated in drug-induced liver injury (DILI), as we previously reviewed.[13] Probing for an endogenous metabolite to compare its level to the baseline in the presence or absence of a drug is one way to detect the CYP3A induction potential of a compound. Measuring the plasma levels of 4β-HC is a cost-effective and minimally invasive approach to studying CYP3A4 and CYP3A5.[11,14] Measurement of this metabolite has also been used to compare the inducibility of CYP3A4 with various drugs, and it has even been applied as a guideline for dosing adjustments.[15] The plasma 4β-HC level has been reported to be a useful parameter for studying CYP3A interactions in nonhuman animals such as rats, mice, and dogs.[16]

Although there has been considerable research on deoxycholic acid and cholesterol metabolism, perhaps the most extensive body of knowledge concerning the role of the CYP3A family with respect to endogenous steroids has come from investigations of the sex hormone testosterone. Testosterone has several metabolic fates, but CYP3A enzymes are responsible for producing the 6β-hydroxy metabolite.[17] This catalysis is widely used to study the effects of potential CYP3A-interacting drugs, owing to the robustness and sensitivity of regioselective testosterone hydroxylation by these enzymes. More recently, plasma levels of 4β-hydroxycholesterol have been used for CYP3A activity probes.[18] Assays for measuring the levels of testosterone metabolites by comparison to those obtained with ketoconazole as a control inhibitor have been developed and optimized as a straightforward means of assessing CYP3A activity.[19] Importantly, CYP3A4 and CYP3A5 have both been shown to catalyze this reaction, although less metabolite is contributed from CYP3A5 when human liver samples are used, owing to the lower hepatic expression of CYP3A5.[20] Probing for 6β-hydroxytestosterone (6β-OHTST) is of particular value when examining samples with diverse populations of CYPs, such as those from liver.[21]

Steroidal derivative endogenous substrates of the CYP3A family also exist. One example was reported by Gupta *et al.*,[22] who demonstrated that CYP3A4 acts upon vitamin D. Although nonselective, CYP3A4 catalyzes the 25-hydroxylation of 1α-hydroxyvitamin D_2 (1α-OHD_2), and, to a lesser extent, 1α-hydroxyvitamin D_3 (1α-OHD_3).[22] Before this finding, this 25-hydroxylation was reported as being catalyzed only by CYP24A1.[23] Although 1α-OHD_2 can be metabolized by CYP3A4, its capacity to act as a sensor of CYP3A activity should not be heavily relied upon, as the metabolite detected in serum does not correlate well with the established 4β-HC metabolite.[24]

2.2. Important exogenous substrates and metabolites

Given the remarkable promiscuity of the CYP3A family, it is not surprising that a broad range of prescription medications can serve as their substrates. These enzymes can metabolize many different classes of compound, each comprising many structurally diverse subsets. The effects of CYP3A–drug interactions vary greatly according to the individual drug, and these consequences need to be studied carefully to address unintended side effects. Chemotherapeutics are one class of drug that is largely implicated in CYP3A interactions.[25] For example, the microtubule-destabilizing vinca alkaloids vincristine, vinblastine, and vindesine are substrates of CYP3A4, CYP3A5, and CYP3A7.[26,27] Other antimitotics of the same parent class, such as the taxanes paclitaxel and docetaxel, are also metabolized by these enzymes.[28,29] Although paclitaxel is subject to CYP3A-mediated hydroxylation at different sites, it has been demonstrated that the 6α product is the primary metabolite.[30] One consequence of this is a 30-fold loss in the cytotoxic activity of the drug.[30] Likewise, docetaxel is metabolized by CYP3A into completely inactive metabolites.[31] Another example within this subclass is cabazitaxel. Although this is a substrate for CYP2C8, CYP3A4 is reported to be primarily responsible for the substantial decrease in the oral bioavailability of the compound.[32] CYP3A-mediated inactivation of pharmaceuticals is clearly a problem with respect to drug efficacy; however, some consequences of CYP3A metabolism are more serious and pose significant health risks to patients via the generation of toxic metabolites.

Tyrosine kinase inhibitors (TKIs) have been adopted as useful compounds for treating various cancers and other diseases. Many approved drugs within this class are subject to CYP3A-mediated biotransformation as their primary metabolic pathway, as reviewed elsewhere.[33] Some TKI metabolites not only lessen the intended effects of the drug, but also exhibit considerable toxicity themselves. Adverse reactions resulting from these metabolites can lower the quality of life for the patient and often necessitate a reduction in the dosage of a drug, thus dampening its intended effect.[34] The human epidermal growth factor receptor/proto-oncogene neu (EGFR/HER-2) inhibitor lapatinib is one example of a compound whose CYP3A4-and CYP3A5-catalyzed metabolites have been implicated in severe drug-induced hepatotoxicity and even in some fatalities.[35] Furthermore, the concentration of lapatinib can be dramatically influenced by the induction or inhibition of CYP3A4 activity—260% increases in the area under the curve (AUC) have been reported when the CYP3A4 activity is inhibited with ketoconazole.[36] Thus, modulating the enzymes responsible can influence the concentrations of harmful metabolites. Another example of CYP3A-mediated hepatotoxicity arising from TKI metabolism is associated with the BCR-ABL inhibitor dasatinib. CYP3A4 oxidizes this compound to produce at least two reactive intermediates with the capacity to covalently bind proteins.[37] Quinone-imine products are formed by further dasatinib oxidation and are partly responsible for the toxic effects. Interestingly, one of the metabolites is active and equipotent to dasatinib itself.[35] The EGFR inhibitor erlotinib is another approved medicine of this class that is a substrate of CYP3A4 and CYP3A5.[38] Erlotinib is approved for non-small cell lung cancer and pancreatic cancer, but its toxicity must be considered before it is given to patients. Skin-related adverse reactions and, more seriously, acute hepatotoxicity have been reported for this compound.[39] Although not explicitly reported as a function of any one metabolite, it is proposed that this toxicity occurs via reactive epoxide and electrophilic quinone-imine intermediates produced largely by CYP3A4.[40] Additionally, it is possible that TKI metabolites produced by the CYP3A pathway induce toxicity by altering gene expression. Microarray profiling has been used to investigate this effect in patients with PDAC who develop erlotinib-associated skin toxicity, possibly potentiated through CYP3A.[41]

Outside the realm of chemotherapeutics, opioids are another class of drugs that are partly metabolized by the CYP3A system. In particular, CYP3A4 has been reported to be a critical mediator of the efficacy of these compounds and of the patient response to many of them.[42] The use of opioids as pain-management tools for patients with cancer also requires careful thought regarding the extensive interplay between these drugs and CYP3A enzymes. The induction or inhibition of opioids by the responsible CYPs may drastically alter the intended analgesic effects. Administering compounds that result in CYP3A4 induction, for example, will markedly reduce the pain-alleviating effects and potentially require higher dosing than would otherwise be expected.[42] One commonly prescribed opioid substrate of CYP3A enzymes is alfentanil, a synthetic opioid that is metabolized by CYP3A4 and CYP3A5 into the primary metabolite noralfentanil.[43] Because of the extensive CYP3A-mediated metabolism of alfentanil, as with several other opioids, modulation of one of the enzymes responsible can produce drastic effects in the context of drug disposition. Indeed, it was reported that the induction of CYP3A4 with rifampicin produced a 3-fold increase in alfentanil clearance.[44] In addition to small molecule modulation, inherent expression differences among CYP3A enzymes resulting from interindividual variability also contribute to this highly variable drug clearance.[44]

2.3. CYP3A modulation by small molecules

The clinical impact of modulating CYP3A enzymes is so significant that the Food and Drug Administration (FDA) recommends testing new drugs for potential CYP3A interactions and doing so by using two structurally unrelated substrates.[45] Moreover, the CYP3A family enzymes can be modulated by various compounds, including FDA-approved drugs, in some fashion-with both overlapping and distinct catalytic activities (Fig. 2). Among the most potent and effective modulators of CYP3A4 and CYP3A5 are azole-containing antifungal compounds. Ketoconazole, a prototypical CYP3A inhibitor, has submicromolar half maximal inhibitory concentration (IC_{50}) values for CYP3A4.[46] Concordantly, ketoconazole-mediated CYP3A inhibition in patients dramatically reduces the metabolism of many drugs that are subject to hepatic clearance. The inhibitory effects of ketoconazole on CYP3A activity in patient populations have far-reaching consequences for drugs intended for various uses, such as diminishing metabolism of the antipsychotic risperidone,[47] the sedative midazolam,[48] the kinase inhibitors fostamatinib and midostaurin,[49,50] and the contraceptive drospirenone,[51] among others. The antifungal isavuconazole is a more moderate inhibitor of CYP3A4 than is ketoconazole, but it still influences the metabolism of CYP3A substrates such as midazolam and norethindrone in patients.[52] Apart from the studies of antifungal effects in humans, several cell-based *in vitro* studies have also demonstrated the ability of these compounds to modulate CYP3A activity and have addressed the responses to specific isoforms by using recombinantly expressed enzymes. For example, Godamudunage *et al.*[53] tested 13 different azole-containing drugs to evaluate their modulation of CYP3A4 versus CYP3A7. Although some of the tested compounds demonstrated CYP3A7 inhibition, the inhibitory effect of these compounds on CYP3A4 was consistently more pronounced.[53] This is of particular clinical relevance because CYP3A4 is expressed in adult liver, whereas CYP3A7 is primarily expressed in neonates. Moreover, when testing nine antifungals for CYP3A4 and CYP3A5 inhibition, Hariparsad *et al.*[54] reported that the commonly prescribed itraconazole, ketoconazole, and miconazole were more inhibitory than were other azole drugs.

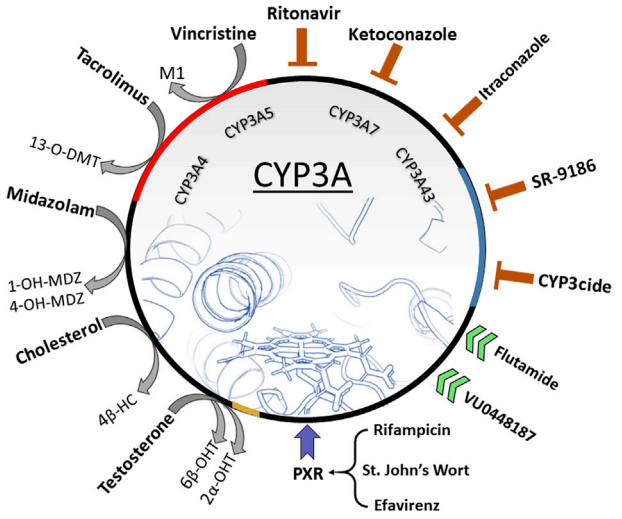

Fig. 2. Effects of various compounds on CYP3A. Several relevant endogenous compounds, pharmaceuticals, or tool compounds are indicated as substrates (gray curved arrows), inhibitors (orange flattened arrows), enzymatic activators (green double arrows), or inducers (purple arrow) of CYP3A. Known selectivity information is indicated along the circle as red (CYP3A5-selective), blue (CYP3A4-selective), yellow (CYP3A7-selective), or black (unknown or non-selective). Abbreviations: PXR, pregnane X receptor; 2α-OHT, 2α-hydroxytestosterone; 6β-OHT, 6β-hydroxytestosterone; 4β-HC, 4β-hydroxycholesterol; 4-OH-MDZ, 4-hydroxymidazolam; 1-OH-MDZ, 1-hydroxymidazolam; 13-O-DMT, 13-O-desmethyltacrolimus; M1, vincristine metabolite 1; CYP3A, cytochrome P450 3A; VU0448187, 5-(4-fluorobenzyl)-2-((3-fluorophenoxy)methyl)-4,5,6,7-tetrahydropyrazolo[1,5-a]pyrazine; SR-9186, 1-(4-imidazopyridinyl-7phenyl)-3-(4′-cyanobiphenyl) urea; CYP3cide, 1-methyl-3-[1-methyl-5-(4-methylphenyl)-1H-pyrazol-4-yl]-4-[(3S)-3-piperidin-1-ylpyrrolidin-1-yl]-1H-pyrazolo[3,4-d]pyrimidine.

Antiretroviral drugs are an example of a class of compounds that contains both inducers and inhibitors of CYP3A. Some drugs in this class can directly bind to and inactivate CYP3A enzymes, whereas others interact with upstream transcription factors and induce CYP3A expression. CYP3A4, for example, is transcriptionally regulated primarily by pregnane X receptor (PXR), but also by other nuclear receptors such as constitutive androstane receptor (CAR), vitamin D receptor (VDR), and hepatocyte nuclear factor 4 alpha (HNF4A).[55,56] Various antiretrovirals induce CYP3A4 by engaging its upstream regulators. For example, efavirenz is an antiretroviral that strongly induces the expression of CYP3A4 via activation of the human transcription factor PXR,[54] whereas atazanavir inhibits CYP3A4.[57] Ritonavir is one of the strongest CYP3A inhibitors in this class, having an IC_{50} of 14 nM, and is even used as a pharmacokinetic boosting agent for other similar compounds because it so effectively halts CYP3A catalysis.[58] Interestingly, the strong inhibitory effect of this antiretroviral can be exploited for other drug classes. For example, it has been proposed to use ritonavir in patients with cystic fibrosis (CF) to inhibit the metabolism of the anti-CF drug ivacaftor and thereby curtail the CYP3A-mediated adverse effects that would otherwise result in reduced efficacy of the latter drug.[59] Cobicistat is often used in a similar manner to ritonavir and acts as an inhibitor for and substrate of CYP3A4.[60] Although both of these drugs modulate CYP3A via potent inhibition, cobicistat has been reported to be more specific for CYP3A, having fewer interactions with other CYPs;[61] accordingly, it has been proposed as an important component of combination regimens for human immunodeficiency virus (HIV) management.[62] Not all antiretroviral drugs modulate CYP3A enzymes equipotently. Although ritonavir is a preferential and potent inhibitor of CYP3A4 and CYP3A5, its inhibition of CYP3A7 is weaker.[63] This information may prove beneficial when treating infants with these compounds. The varying effects and wide potential for CYP3A-mediated adverse interactions when antiretrovirals are used in combination with other medications necessitate careful consideration when prescribing these drugs.

Apart from conventional medications that act as small molecule modulators of CYP3A, natural products present in common foods can also exert such effects. The best-known example was discovered nearly 30 years ago when it was reported that grapefruit juice changed the bioavailability of the antihypertensives felodipine and nifedipine.[64] It is now accepted that more than 80 commonly prescribed drugs interact unfavorably with grapefruit juice, resulting in serious adverse reactions such as rhabdomyolysis, myelotoxicity, nephrotoxicity, and respiratory depression.[65] The observed consequences are a direct result of potent inhibition of CYP3A enzymes (primarily CYP3A4, according to reports), which results in a failure to metabolize these compounds and, thus, in the accumulation of toxic concentrations in the body. Bergamottin is the furanocoumarin in grapefruit that is responsible for CYP3A4 inhibition; importantly, it causes mechanism-based (or “suicide”) inactivation.[66] This irreversible inhibition is caused by covalent modification of CYP3A4 and contributes more substantially to a reduction in the available enzyme when compared to a reversible inhibitor.[67] In addition to the unfavorable increase in the bioavailability of many drugs, grapefruit juice also inhibits the metabolic activation of CYP3A prodrugs—two examples thus affected are the blood thinners clopidogrel and prasugrel.[68,69] Other natural products originating from fruits and vegetables have been reported as modulators of CYP3A enzymes. Cabbage and onion juices, for example, were reported to be inhibitors of CYP3A4 activity in both biochemical- and cell-based experiments.[70] Bael fruit,[71] evodia fruit,[70] goji berry,[72] and starfruit all contain small molecule CYP3A inhibitors that lead to various adverse drug interactions.[73] Studies have also implicated CYP3A enzymes in herb-drug interactions. These studies help to explain some of the toxicities associated with traditional medicines and modern drugs. Cat's claw and peppermint oil are examples of highly potent CYP3A4 inhibitors that may interfere with prescription medications.[74] Schisandra chinensis (magnolia vine) is a plant used in traditional Chinese medicine that contains CYP3A4 and CYP3A5 inhibitors that lead to dramatically higher bioavailability profiles of certain CYP3A-interacting drugs.[75] Furthermore, natural products from foods and herbs can also be inducers of CYP3A enzymes. St. John's wort is one such example; hyperforin, a component of St. John's wort, acts as a potent inducer of CYP3A4 expression by activating its upstream transcriptional regulator PXR.[76,77] The use of this herb in patients taking prescription medications presents a serious safety concern because of the high potential for interactions.[78] The routine consumption of food products containing CYP3A modulators may account for observed differences in drug efficacy and toxicity, beyond what can be explained by interindividual variability.

One interesting form of CYP3A modulation is that of direct enzymatic activation, whereby a compound binds to an enzyme and induces a level of enzymatic activity that is higher than the basal levels. Modulators in this category are generally considered to be allosteric regulators that bind outside or distal from the classical heme-containing active site in a manner that structurally enables increased catalysis. A few researchers have hypothesized the existence of peripheral ligand-binding sites for CYP3A4.[79,80] The compound 5-(4-fluorobenzyl)-2-((3-fluorophenoxy)methyl)-4,5,6,7-tetrahydropyrazolo[1,5-a]pyrazine (hereafter referred to as VU0448187) is an activator of CYP3A4 and CYP3A5 that increases midazolam hydroxylation activity by more than 100-fold compared to the baseline level.[81] Interestingly, the activity of VU0448187 is reportedly substrate dependent; it affects the hydroxylation of midazolam, but not that of testosterone and progesterone.[82] As

VU0448187 is intended as a ligand of metabotropic glutamate receptor 5 ($mGlu_5$), there is a possibility of drug–drug interactions.[81] Similarly, the prescription androgen receptor antagonist flutamide and its metabolite 2-hydroxyflutamide are also CYP3A heterotropic activators.[83] Flutamide can induce CYP3A4 and CYP3A5 midazolam hydroxylation activity that is more than 100% higher than the basal level and can result in a dramatic increase in the CYP3A substrates nifedipine and amiodarone.[83] A particularly interesting case of CYP3A activation involves the platelet aggregation inhibitor ticagrelor. This compound exhibits an intriguing bimodal modulation of CYP3A activity, whereby the hydroxylation of midazolam to 4-hydroxymidazolam is mildly inhibited but the conversion to 1-hydroxymidazolam is mildly increased.[84] This may contribute to the adverse effects seen when patients are given ticagrelor with CYP3A inducers.[82] Progesterone has also been proposed as a CYP3A4 allosteric activator,[85] but little work has been done to investigate its effect on drug–drug interactions as a direct result. Isoform-selective allosteric activation was reported with the TKI icotinib, which appears to activate CYP3A5 but not CYP3A4.[86] Small molecule enzymatic activators of CYP3A family members represent yet another potential source of drug–drug interactions. The unintended increase in CYP3A metabolic activity (and, thus, the unintended increase in CYP3A-mediated metabolism of drugs) means that caution is warranted when drugs are taken alongside these activators.

2.4. Selective catalysis and substrate recognition

Members of the CYP3A family are commonly regarded as having enough overlapping substrate specificity to justify considering them as a single enzyme. Indeed, many research studies do not differentiate between the isoforms and frequently use annotations such as “CYP3A4/5.” The tissue-specific expression and overall abundance of each enzyme in the CYP3A family are occasionally taken into account, but the individual metabolic capabilities of these enzymes receive much less consideration. However, several isoform-specific or isoform-preferential reactions are known to occur within the CYP3A family, and some have clinical relevance. In recent years, important tool compounds have been developed that exploit subtle differences between CYP3A4 and CYP3A5, enabling evidence of isoform-specific catalytic activities to be obtained. In 2012, two separate groups published the first reports of CYP3A4-selective inhibitors. 1-(4-Imidazopyridinyl-7phenyl)-3-(4′-cyanobiphenyl) urea (SR-9186) is a CYP3A4 inhibitor demonstrating 1000-fold selectivity over CYP3A5,[87] whereas 1-methyl-3-[1-methyl-5-(4-methylphenyl)-1H-pyrazol-4-yl]-4-[(3S)-3-piperidin-1-ylpyrrolidin-1-yl]-1H-pyrazolo[3,4-d]pyrimidine (CYP3cide) is a mechanism-based (irreversible) CYP3A4-selective inhibitor.[88] At present, there is no selective CYP3A5 inhibitor available. Although there is a clear need for an isoform-selective CYP3A5 inhibitor, it has been suggested that the development of such a compound presents a considerable challenge.[9,89,90] This is probably based on the observation that when a compound tends to inhibit both CYP3A4 and CYP3A5, it is almost always CYP3A4 that is more potently inhibited. However, a probe compound for selectively measuring CYP3A5 does exist. In 2014, the N-oxide metabolite of the phosphodiesterase inhibitor T-1032 (hereafter referred to as T5NO) was the first compound to be identified as a highly selective metabolite catalyzed by CYP3A5, demonstrating greater than 100-fold selectivity over CYP3A4.[91] Although T-1032 is metabolized by CYP3A4, CYP3A5, and CYP2C8, the T5NO metabolite is produced almost exclusively by CYP3A5. These tool compounds have proved instrumental in the delineation of the discrete contributions of CYP3A isoforms.

Vincristine was one of the earliest compounds reported to be catalyzed preferentially by a CYP3A isoform. Dennison *et al.*[7] tested various recombinantly expressed CYPs and showed that vincristine oxidation was significantly more efficient in the context of CYP3A5 as compared to CYP3A4. In a subsequent study, the same authors further characterized the CYP3A5-mediated 14-fold metabolite increase, reporting that genetic polymorphisms of CYP3A5 play major roles in the biotransformation of this compound.[92] Moreover, when CYP3A5 is inactive because of genetic polymorphisms, vincristine-induced toxicity is heightened, further suggesting that CYP3A5 is critical for the clearance of this drug.[93] Importantly, there is evidence that the CYP3A5 genotype is not the only causative factor associated with vincristine toxicity.[94] No structure for CYP3A5 bound with vincristine has yet been solved, but molecular dynamics simulations have been employed to help explain its preferential catalysis,[95] and the researchers who conducted those simulations hypothesized that vincristine binds to CYP3A5 in an orientation different from that of CYP3A4, enabling it to interact more tightly with critical active-site residues. Another well-characterized association between CYP3A5 and selective catalysis is that observed with the immunosuppressant tacrolimus. Patients receiving organ transplants are prescribed this drug to suppress their immune systems to avoid rejection of the transplant. Both CYP3A4 and CYP3A5 can metabolize tacrolimus into 13-O-tacrolimus, but the catalytic efficiency is 64% higher for CYP3A5.[96] As with vincristine, genetic polymorphisms of CYP3A5 (particular those that classify patients as “expressors” versus “non-expressors”) contribute dramatically to the efficacy of the drug and provide the basis for dosing adjustments.[97] Because tacrolimus is one of the few drugs that is catalyzed preferentially by CYP3A5, the expression levels of the functional CYP3A5 isoform of a patient must be carefully considered when prescribing this drug. Naturally, nonselective inhibition of CYP3A5 via ketoconazole or cyclosporine A also affects tacrolimus biotransformation and, thus, the proper dosage.[8]

When panels of compounds are tested for their CYP3A4 and CYP3A5 inhibitory potential, the general pattern is that if a compound inhibits CYP3A4 then it probably also inhibits CYP3A5.[98] Similarly, most substrates of CYP3A4 are also substrates of CYP3A5. Although the potencies usually differ (with most compounds being more potent for or more efficiently catalyzed by CYP3A4 than CYP3A5), changes greater than 2- to 5-fold are not commonly observed. Because CYP3A4 is the primary CYP3A enzyme expressed in liver, side-by-side comparisons with CYP3A5, using recombinantly expressed enzymes, have been only infrequently conducted. However, one CYP3A4-selective compound was reported in 2016, when it was demonstrated that gomisin A is metabolized nearly 13 times more efficiently by CYP3A4 than by CYP3A5.[99] CYP3A4 preferentially catalyzes the hydroxylation of this natural compound to 8-hydroxygomisin A, and this product was not detected when other human CYPs were tested. Interestingly, gomisin A is also reported to be an inhibitor of CYP3A4.[100] Compounds serving as substrates that are also inhibitors at clinically relevant concentrations may help to provide insights into modes of isoform-selective catalysis and/or inhibition of the human CYP3A family. Moreover, gomisin A is of additional interest because of its hepatoprotective effects, which may be directly related to interplay with CYP3A4.[101,102] Another important example of a compound that is selectively catalyzed by CYP3A4 is luciferin-IPA, which has been developed by Promega for use in drug-development assays.[103] This is a “pro-luciferin” that is selectively metabolized into D-luciferin by CYP3A4, with very minimal cross-reactivity from other CYP3A members and none from various other human CYPs.[103] Adding luciferase to the system yields a luminescent signal, and this technique has been routinely used as a robust measure of various biological processes, such as

cytotoxicity.[104] Leveraging this in the context of CYP3A, the signal from luciferin-IPA being converted to D-luciferin (and ultimately luminescence) is directly proportional to the CYP3A4 activity, making this method suitable for high-throughput drug-development screens.[105] In systems that express multiple CYP3A family members, isoform-selective substrates such as these are important tools for uncovering the individual roles of a given isozyme.

3. Structural perspectives

3.1. Secondary structures and flexibility

Several crystal structures of human CYP3A4 have been solved and deposited in the Protein Data Bank (PDB, https://www.rcsb.org). These structures serve as informative tools with which to further the understanding of the enzymatic function of this molecule. The flexibility of CYP3A4 has been well established and is an essential part of its ability to so promiscuously recognize and bind compounds.[106] The ligand-accessible volume of the CYP3A4 binding pocket is estimated to be very large at 520 $Å^3$.[107] It can bind a hugely diverse set of ligands (*i.e.*, compounds that bind to the CYP3A4 binding pocket) and even exhibits multiple conformations itself.[108] CYP3A4 and CYP3A5 have been described as having the highest catalytic promiscuity among the major human CYPs.[109] Some of the structural diversity of the compounds that bind to CYP3A4 can be observed in the various crystal structures. Although structurally diverse, these ligands also have varying functions. Compounds that exemplify the structural and functional diversity of CYP3A4-binding ligands, as determined using X-ray crystallography, include the endogenous hormone progesterone,[110] the antibiotic erythromycin,[111] the antifungal ketoconazole,[111] the antiretroviral ritonavir,[112] the dopamine promoter bromoergocryptine,[109] and the sedative midazolam (Fig. 3).[113] Interestingly, the cavity can adapt to accommodate multiple ligands at once, as seen with ketoconazole and ritonavir. It can also adopt a conformation that may present a noncanonical binding site (as seen with progesterone). The most striking differences in flexibility when binding various ligands occur in the F–F′ region.[114] This change is apparent when the ligand-free states of CYP3A4 (in the absence of ligand or coordinated to a water molecule) are compared with the structure that has two molecules of ketoconazole bound and an expanded active site (Fig. 4A).[111,115,116] The F–F′ region is expanded outward as a result of the inherent flexibility of the secondary structure lining the roof of the pocket. This culminates in an expanded state that can accommodate two molecules of ketoconazole. The ability of CYP3A4 to conform to diverse molecules is a direct function of this secondary structure flexibility.

Only one crystal structure has been solved for CYP3A5. In 2018, Hsu *et al.*[117] were the first to crystalize CYP3A5, bound with the potent inhibitor ritonavir. This was the first concrete evidence that although CYP3A4 and CYP3A5 have largely homologous overall secondary structures, certain residues lining the binding pocket of CYP3A5 do indeed confer a unique shape. Amino acid residues in the F–F′ region are positioned slightly differently in the two enzymes. Consequently, the CYP3A4 binding pocket has a comparatively shorter and more horizontal shape when compared to the CYP3A5 binding pocket, whereas the roof of the latter pocket is higher and narrower than that of the CYP3A4 pocket.[117] Subtle changes such as these probably contribute to the differences that constitute the basis for isoform-selective inhibition and catalysis, where observed. On comparing the CYP3A5 structure to CYP3A4 crystallized with the same ligand,[118] it is apparent that ritonavir can extend into the roof of the CYP3A5 binding pocket but is blocked by the position of F213 in CYP3A4 (Fig. 4B). It is, therefore, reasonable to suppose that compounds that extend into this available region of CYP3A5 could be developed as selective substrates or inhibitors. This structure presents an opportunity to exploit key differences between these two enzymes to further understand their selectivity.

3.2. Contrasting active sites and the consequences thereof

The in-depth exploration of active-site differences of CYP3A enzymes and the resulting biological consequences is just beginning. At present, no crystal structure exists for CYP3A7 or CYP3A43. However, kinetic analyses and *in silico* studies can provide insights into the selectivity of these enzymes and other CYP3A family

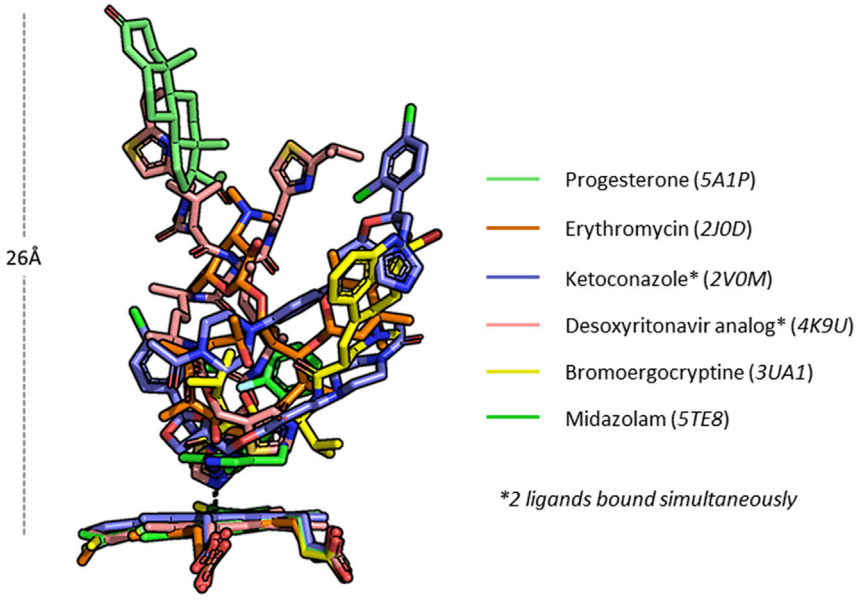

Fig. 3. Promiscuity of the CYP3A4 ligand-binding pocket. The crystal structures of CYP3A4 were obtained from PDB and aligned to illustrate the structural diversity of ligands capable of binding to CYP3A4. Progesterone (PDB: 5A1P) is shown in light green, erythromycin (PDB: 2J0D) in orange, ketoconazole (PDB: 2V0M) in blue, desoxyritonavir analog (PDB: 4K9U) in pink, bromoergocryptine (PDB: 3UA1) in yellow, and midazolam (PDB: 5TE8) in green. Asterisks (*) indicates that two molecules of the same ligand are bound in the respective structures.

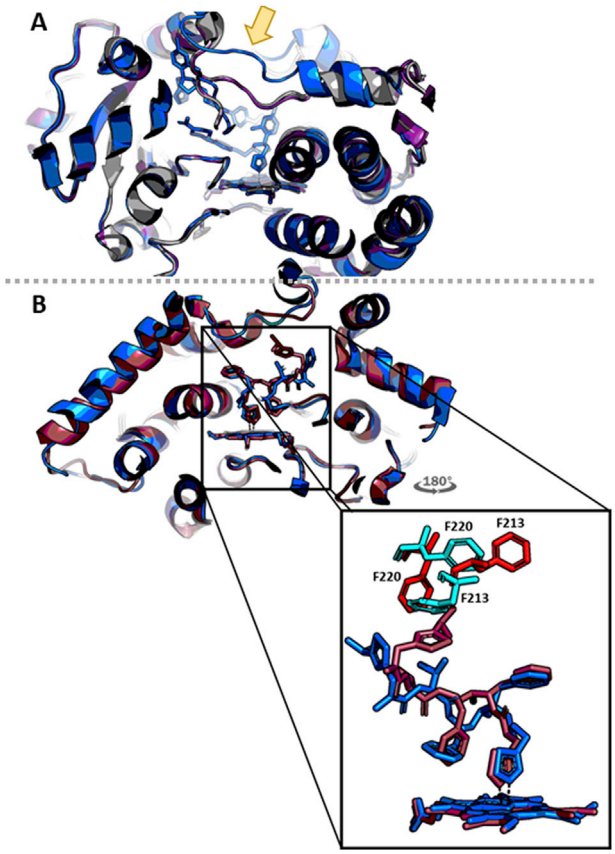

Fig. 4. Structural differences in CYP3A conformations. (A) CYP3A4 demonstrates flexibility in the active site. Ketoconazole-bound (PDB: 2V0M) (blue), water-bound (PDB: 4I3Q) (purple), or ligand-free (PDB: 1TQN) (gray) structures of CYP3A4 are overlaid. The flexibility of the F–F′ region at the roof of the binding pocket in the ketoconazole-bound structure is indicated by the yellow arrow. **(B)** Differences in key active-site residues in CYP3A4 and CYP3A5 contribute to the differential binding modes of ritonavir. Ritonavir-bound CYP3A4 (PDB: 3NXU) (blue) superimposed with ritonavir-bound CYP3A5 (PDB: 5VEU) (raspberry) demonstrates slightly different ligand-binding poses. The magnified panel shows the residues responsible for this difference in CYP3A4 (cyan) versus CYP3A5 (red).

members. It was previously reported that these enzymes exhibit regioselective and stereoselective differences in testosterone metabolism.[119] It is well established that CYP3A family members are responsible for testosterone metabolism.[17] Interestingly, however, Nebert *et al.*[120] investigated the apparent differences in the production of 6β-hydroxytestosterone (6β-OHT), 2α-hydroxytestosterone (2α-OHT), and 2β-hydroxytestosterone (2β-OHT) and proposed that active-site differences between CYP3A4/5 and CYP3A7 contribute to testosterone binding in a position that favors the 2α-OHT metabolite. The differences in the binding mode and resulting metabolite suggest that the 2α-OHT product is important in the fetal environment, as CYP3A7 is the primary fetal CYP3A enzyme. This could also serve as an example of how subtle differences between CYP3A catalytic sites confer different and potentially biologically relevant activities. Another example of differential effects among CYP3A enzymes that result from differences in their active sites is seen with the lignan compounds gomisin C and gomisin G.[75] The inhibition profiles of these compounds appear to be different for CYP3A4 versus CYP3A5, with consequent varying effects on the substrates midazolam, testosterone, and nifedipine. This is intriguing because gomisin C and gomisin G share high structural similarity and have the same molecular weight. When *in silico* modelling studies were performed, docking of these compounds revealed an interesting CYP3A5-exclusive hydrogen-bonding interaction.[75] Importantly, these compounds also showed different capacities for interaction with heme groups. It is plausible that the minor differences between CYP3A active sites, which were previously considered trivial, are responsible for how certain molecules fit and interact differently within these enzymes and, thus, elicit differing biological consequences.

4. Similarities and differences between CYP3A enzymes and other CYPs

4.1. Comparisons to other human CYPs

Although the CYP3A family comprises the CYPs that metabolize the widest range of compounds, other enzymes in this superfamily also play critical roles in drug metabolism and response to stimuli. Humans have 18 distinct CYP families, and it is accepted that the most abundant and biologically relevant members hail from CYP1, CYP2, CYP3, and CYP4.[120] These families do share some redundancy, but specific roles in metabolism and disease progression have been reported for various enzymes within them. Moreover, differences in their tissue-specific expression patterns have provided clues to their intended functions and clinical relevance. The CYP1 family is one of the primary CYP families expressed in lung.[121] Interestingly, CYP3A5—but not CYP3A4—is expressed in lung with the CYP1 family.[121] Within the CYP1 family, CYP1A1, CYP1A2, and CYP1B2 catalyze the activation of polycyclic aromatic hydrocarbons,[122] and they have recently been reported to possess pro-cancer effects.[123] The inhibition of CYP1B1 has recently been reported to have anti-angiogenic activities, and it has been suggested to be an important target for anticancer strategies.[123,124] Unlike the CYP3A family, which is transcriptionally regulated by PXR, CYP1 is controlled the aryl hydrocarbon receptor (AHR).[125] The CYP2 family includes some of the primary CYPs expressed in endothelium, myocardium, and kidney,[126] but members can be found in other tissues too. CYP2B6 is among the most clinically relevant CYP2 enzymes; it is proposed to metabolize around 2%–10% of clinically prescribed drugs.[127] CYP2B6 is the primary CYP inhibited by drugs such as artemisinin, bupropion, cyclophosphamide, and ketamine.[128] The constitutive androstane receptor (CAR), which is known to have overlapped regulation and function with PXR, transcriptionally controls the expression of this enzyme. Like the members of the CYP3A family, CYP2B6 is highly polymorphic and displays population disparities in its genotype.[128] The CYP4 family comprises 13 members that are primarily implicated in the metabolism of eicosanoids and fatty acids, most notably through their ω-hydroxylase activity.[129] This family has been specifically implicated in the progression of fatty acid–linked diseases and certain cancers, and its members represent an important class of drug targets.[130] Like all CYPs, these enzymes have a heme group that is important for catalytic activity. However, CYP4 enzymes generally prefer to covalently bind their heme group, whereas the CYP3A enzymes catalyze a reaction and turn over the product, leaving them ready to repeat the process.[131] CYP4F2 and CYP4F12 are expressed in liver and are currently being investigated as biomarkers for hepatocellular carcinoma.[132]

4.2. Evolutionary, structural and functional comparisons of CYP3A and bacterial CYPs

Evolutionary and phylogenetic studies on CYPs show that these enzymes evolved in archaea approximately 3000 million years ago,[133] whereas the present human CYPs, including the CYP3A family, may date back to around the Devonian and Silurian periods

approximately 400 million years ago—a period of co-evolutionary "warfare" involving plants and animals as a result of the increasing atmospheric oxygenation and the detoxification of plant toxins.[133] As a result, isoforms of CYPs span the evolutionary spectrum from archaea through prokaryotes (including bacteria) to eukaryotes (plants and animals), always encoding the consensus 10–amino acid signature motif that includes the heme iron–ligating cysteine residue in the format FxxGxxxCxG.[134,135] The mammalian CYP3A enzymes (and other eukaryotic CYPs) are predominantly integral monotopic membrane proteins anchored to the membrane of the endoplasmic reticulum via an N-terminal membrane-spanning helix, where they interact with their redox partner enzymes, the flavin adenine dinucleotide (FAD)- and flavin mononucleotide (FMN)-binding NADPH-cytochrome P450 reductase (CPR).[136,137] The CYPs catalyze the monooxygenation of substrates via a reductive scission of oxygen bound to their active-site heme iron by the timely provision of two electrons that facilitate oxygen activation and the generation of highly reactive oxygen species and the final insertion of a single oxygen atom into the substrate.[137] In contrast, bacteria have soluble CYPs that are cytosolic and require interaction with other soluble partner proteins (usually ferredoxins and ferredoxin reductases) in order to catalyze similar monooxygenation of substrates while using a distinct redox apparatus. Nevertheless, even though the two forms of CYPs are located in different cellular environments in bacteria and humans, virtually all CYPs catalyze the reductive scission of molecular oxygen, resulting in the oxidation of the substrate in a similar fashion in these different life forms.[138]

CYPs in all life forms exhibit similar structural folding and conformation. Although there may be significant differences in their amino acid sequences, they are all globular proteins that are assembled into what is commonly called the "P450" fold. This tertiary structure of CYPs is largely due to the formation of α-helices and a small domain with a significant number of β-sheets.[139,140] Despite these sequence disparities, each CYP secondary structure contributes amino acid residues that are critical for the folding, flexibility/stability, and catalysis needed for the different roles of the CYPs. These CYP secondary structures are generally composed of approximately 80% helices, 15% β-sheets, and 5% loops and turns.[139,140] However, there is significant variation in the construction of the helices for the various CYPs, with an especially notable difference being apparent between the shorter ones and those at the surface of the enzyme, such as helices A, B, G, and K, which vary in length among CYPs and are generally assigned with a gap representing two parts of the helix linked by a short peptide. This feature can clearly be seen by comparing CYP3A4 (or CYP3A5) with the overly characterized bacteria CYP 102A1 (P450 BM3) from *Bacillus megaterium*: CYP3A4 possesses a G′–G and F′–F helix linked by a short amino acid peptide, whereas P450 BM3 contains a single helix approximately 17 amino acids in length.[139,141] A hypothetical explanation for the prevalence of these "gapped" flexible helices in CYP3A enzymes could be their involvement in active-site cavity flexibility and facilitation of the CYP3A promiscuity for various large substrates. Indeed, the members of the CYP3A family (particularly CYP3A4) metabolize diverse endogenous substrates (steroids) and exogenous substrates (xenobiotics) when compared to other steroid-oxidizing human and bacteria CYPs, making them prodigious enzymes within the CYP superfamily.[142]

The physiologic roles of the human CYP3A and bacterial CYPs also vary slightly, as the CYP3A family mainly participates in catabolic activities involving xenobiotic metabolism, leading to the excretion of drug compounds, whereas most bacterial CYPs are orphan enzymes with no known activity or identified substrates.[143] However, those bacterial CYPs with known functions catalyze reactions ranging from the catabolism of carbon compounds to sustain the viability of the bacterium (as in the catabolism of host cholesterol by mycobacteria using CYP125A1 and CYP142A1) to anabolic processes such as the biosynthesis of useful bioactive secondary metabolites, including antifungals, antiparasitics, and anticancer agents.[143,144] Typical scenarios include the oxidation of polyketides, *e.g.*, hydroxylating the erythromycin precursor 6-deoxyerythronolide B, and the epoxidation by CYP EpoK of epothilones C and D into epothilones A and B, respectively, which are popular anticancer agents.[145] Although there may be some differences between the CYP3A and bacterial CYPs with respect to their physiologic roles, there appears to be a small consensus among the CYPs that involves metabolizing a steroid/hormone scaffold. Typical examples of this are the inactivation of testosterone to its 6β-hydroxylated metabolite by human CYP3A5, the cholesterol hydroxylase activities of CYP125 and CYP142 in mycobacteria, and the lanosterol 14α-demethylase activity of CYP51 across all organisms (bacteria and eukaryotes). In addition, all CYPs, whether bacterial or eukaryotic, are potently inhibited by various azole antifungal compounds (including clotrimazole, econazole, and ketoconazole), mainly via their competitive ligation to the heme iron moiety present in all CYPs.[146,147] This is further evidence in support of the evolutionary theory that although these CYPs (including the CYP3A family and other bacterial CYPs) are present in different organisms and use distinct forms of redox apparatus for substrate oxidation, they all evolved from a single ancestral lineage millions of years go.

5. Discussion and further perspectives

It is clear that CYP3A enzymes are critical for endogenous metabolism, xenobiotic response, the mediation of drug–drug interactions, and other important biological processes. However, less clear has been the discrete role that a given enzyme in this family might play and whether that role was of any biological importance. The recent efforts to delineate isoform-selective roles of CYP3A enzymes have shown that interesting and clinically relevant functions exist within the vast overlap. CYP3A enzymes are affected in different ways by various compounds, ranging from endogenous metabolites used as biomarkers to inducers that weaken intended drug effects (Fig. 2). Comparing these enzymes in the context of structure, expression, substrate recognition, activity modulation, and catalytic efficiency has yielded insights into the most important differences. It has now been shown that the active sites of CYP3A4 and CYP3A5 have different shapes. It is likely that many compounds can be recognized by both isoforms because their active sites can usually conform to fit the various shapes. However, certain compounds demonstrate that there are scenarios in which compounds bind and confer either preferential catalysis or (in the case of CYP3A4) selective inhibition. These compounds appear to exploit key differences to elicit the observed selectivity. The identification of isoform-selective inhibitors of CYP3A4 suggests that it might also be possible to selectively inhibit CYP3A5, although given the generally higher inhibition potential of CYP3A4, this is likely to be a challenge. Nevertheless, examples such as vincristine and tacrolimus prove that at least some small molecules can be metabolized preferentially by CYP3A5, which is good news in the context of developing a selective CYP3A5 inhibitor. The preferential catalysis of 2α-OHT by CYP3A7 suggests that the latter enzyme may play important roles in development and perhaps even in disease progression. The distinct functions and drug-interaction profiles of CYP3A4, CYP3A5, and CYP3A7 warrant a further characterization of the metabolic capability of CYP3A43, which has been little studied so far. Furthermore, and beyond the scope of this review, genetic polymorphisms within the CYP3A family may represent an extra layer of regulation by changing the substrate recognition or

inhibition potential of these enzymes. Elaboration on what has been reported so far regarding CYP3A selectivity is warranted to obtain a more complete understanding of the biological contributions of each enzyme and how they can be exploited in medicine.

Authors' contributions

W. C. Wright drafted all contents except Section 4.2, and J. Chenge drafted Section 4.2. W. C. Wright generated figures. W. C. Wright, J. Chenge, and T. Chen reviewed, edited, and finalized the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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