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Cytochrome P450 Enzyme Metabolites in Lead Discovery and Development

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Abstract

The cytochrome P450 (CYP) enzymes are a versatile superfamily of heme-containing monooxygenases, perhaps best known for their role in the oxidation of xenobiotic compounds. However, due to their unique oxidative chemistry, CYPs are also important in natural product drug discovery and in the generation of active metabolites with unique therapeutic properties. New tools for the analysis and production of CYP metabolites, including microscale analytical technologies and combinatorial biosynthesis, are providing medicinal chemists with the opportunity to use CYPs as a novel platform for lead discovery and development. In this review, we will highlight some of the recent examples of drug leads identified from CYP metabolites and the exciting possibilities of using CYPs as catalysts for future drug discovery.

Keywords

cytochrome P450; metabolites; drug discovery; natural products; 4-hydroxyatorvastatin; metabolizer phenotype; 14-hydroxyclearithromycin; 6-hydroxybuspirone; fexofenadine; microscale microfluidics

1. INTRODUCTION

The cytochrome P450 (CYP) enzyme superfamily is well known for its role in oxidative drug metabolism^{1–4}, however in recent years there has been a growing interest in the identification of novel therapeutic agents from CYP metabolites. Utilizing their unique oxidative chemistry^{5,6}, CYPs are able to catalyze a wide variety of unusual reactions on small-to-medium-sized organic substrates that can lead to new chemical entities with novel pharmacological activity. CYP enzymes play major roles in plant biosynthetic pathways generating many natural products that have been developed into potent therapeutic agents^{7,8}. Additionally, several of the drugs derived from natural products are further transformed by drug metabolizing CYPs *in vivo* into biologically active metabolites, some of which are more potent than the parent compound⁹. More recently, microscale analytical technologies

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have been employed for the generation and identification of CYP metabolites as lead compounds¹⁰. In conjunction with the development of bioreactor technology, whereby CYP oxidative transformations may be scaled-up for quantitative production of metabolites, this has ushered in the possibility of utilizing CYPs as a platform for lead discovery and development^{10,11}. This review will highlight some of the recent examples of drug leads identified from CYP metabolites and the intriguing possibilities of using CYPs as catalysts for future drug discovery and development.

2. IDENTIFICATION OF CYP-MODIFIED NATURAL PRODUCTS AS DRUG LEADS

A large variety of natural products that have been developed into successful drugs contain CYPs in their biosynthetic pathways. These include antibiotic, antimitotic, antineoplastic, antihypertensive, and antiarrhythmic agents⁴. Many of these compounds are secondary metabolites that are involved in plant or microbial defense pathways^{4,12}. The unique oxidative chemistry provided by CYPs allows tailoring specific functionalities onto complex carbon skeletons to fine tune their biological activities. In this way, millions of years of chemical warfare between microbes, plants, and animals have produced chemical entities that are exquisitely specific for their targets. Only recently have concerted efforts been made to identify new lead compounds from known CYP biosynthetic pathways involved in the generation of natural products, yet these may prove promising in the years to come. A few examples from a variety of classes are illustrated below.

2.1 Antineoplastic agents

The potent antimitotic agent Taxol (paclitaxel), originally isolated from endophytic fungi inhabiting the bark of the Pacific yew tree (*Taxus brevifolia*), is thought to have up to 14 CYPs involved in its biosynthetic pathway^{12,13}. Initially, there were significant concerns regarding obtaining enough of the compound due to the ecological impact of harvesting the trees. These concerns were alleviated when a commercial scale semi-synthetic route, based on extraction from yew pine needles instead of the bark, was identified¹⁴. Production now relies on large scale plant cell fermentation with the paclitaxel-producing fungus *Penicillium raistrickii*¹⁵. Despite Taxol's wide spread use, problems with off-target effects and the potential for resistance has led to a desire to identify new analogs. One approach was to clone several of the paclitaxel pathway CYPs for expression in the yeast *Saccharomyces cerevisiae*, in an attempt to both increase yield and produce novel analogs through combinatorial biosynthesis¹⁶. A total of 8 of the 14 CYPs thought to be involved in the pathway were cloned and expressed in *S. cerevisiae* and this allowed for production of baccatin III, an intermediate in paclitaxel biosynthesis that could function as a precursor for the semi synthesis of novel paclitaxel analogs¹⁶.

In another case, an alternative retrometabolic approach was used by Guengerich and colleagues to identify novel chemotherapeutic agents based on a previously known pharmacophore¹⁷. The serendipitous discovery that several human CYPs are able to metabolize indole to indigo and indirubin led to the hypothesis that they might also be able to generate lead compounds for tyrosine kinase inhibition, since indole is a known

pharmacophore for many of these enzymes¹⁸. Guengerich and colleagues added a variety of commercially available substituted indole compounds to bacterial cultures expressing various human CYP2A6 mutants generated by directed evolution¹⁹. Extracts from these cultures were screened against the kinases CDK1, CDK5, and GSK-3b, and from these initial screenings, they were able to identify several indirubin-based inhibitors that were an order of magnitude more potent than indirubin itself, and characterize their individual structures using ¹H NMR¹⁹. An approach such as this, employing enzyme mutagenesis and enzymatic coupling to produce novel compound libraries of previously known pharmacophores, may be of particular benefit for scaffolds which are synthetically difficult.

2.2 Antiprotozoal agents

The most profound advancement in the treatment of malaria in recent decades has been the development of artemisinin, a sesquiterpene lactone endoperoxide isolated from *Artemisia annua* spp. (Chinese wormwood)⁸ (Figure 1).

This has generated significant interest in cloning the entire biosynthetic pathway for expression in a compliant heterologous host, such as *S. cerevisiae*. Keasling and colleagues were successful in modifying the yeast mevalonate pathway and introducing the genes encoding amorphaadiene synthase and CYP71AV1 from *A. annua*²⁰. In this modified yeast strain, they demonstrated significantly enhanced production and cellular export of artemisinic acid, the direct precursor to artemisinin²⁰. In a subsequent study, after engineering a plant CYP reductase and a second CYP in the pathway from *A. annua*, they were able to produce the final artemisinin product in ~20-fold increased yield, reducing the production cost dramatically²¹.

2.3 Antifungal agents

One of the more efficacious agents used to treat systemic fungal infections is amphotericin B, a polyene secondary metabolite isolated from *Streptomyces nodosus*²² (Figure 2). Its primary mechanism of action is to sequester ergosterol, an important component of fungal cell membranes. However, toxicities resulting from off-target effects in host cell membranes have limited its use²². Previous work had demonstrated that diminishing the negative charge on the exocyclic carboxyl group significantly reduced toxicity²³. Carmody and colleagues thus created a series of *S. nodosus* mutants with targeted deletions in the *amphN* CYP gene locus, a CYP with known tailoring function in the production of amphotericin B, to generate amphotericin analogues where the exocyclic carboxyl groups were substituted by methyl group functionalities²⁴ (Figure 2). These analogs retained antifungal activity while exhibiting reduced hemolytic toxicity. A future effort in this area might focus on other structural perturbations of the molecule utilizing the principals of combinatorial biosynthesis to generate analogs with reduced toxicity and improved efficacy.

Another successful antifungal agent, griseofulvin, first isolated from the mold *Penicillium griseofulvum*, has seen additional interest lately due to its anti-proliferative effects in certain cancer cell model systems²⁵. A recently identified CYP in its biosynthetic pathway has been shown to be essential for formation of the grisan scaffold²⁶. Using this enzyme, along with certain others involved in the biosynthetic pathway, Cacho and colleagues were, for the first

time, able to synthesize griseofulvin entirely *in vitro* from acetyl-CoA and malonyl-CoA feedstocks²⁶. Understanding the role of the CYP in the orcinol and phloroglucinol ring coupling reactions opens up the possibility of creating griseofulvin analogs that may have useful applications as antifungal or antineoplastic agents. A number of exciting technologies to combine CYP active metabolite generation with structural elucidation and activity studies have recently surfaced, all of which will no doubt be a boon for novel lead discovery^{10,27}.

3. IDENTIFICATION OF PHARMACOLOGICALLY ACTIVE METABOLITES OF KNOWN DRUGS

Since the early days of the study of drug metabolism, it's been known that metabolites can be pharmacologically active^{28,29}. Probably the most classic, and earliest, example of this phenomenon was the discovery of the antibiotic sulfanilamide as a metabolite of the drug Prontosil³. Subsequently, the strategy of developing prodrugs most commonly took advantage of metabolism to release an active form of a drug from a pharmacologically inactive precursor³. However, later studies also identified biologically active metabolites from *active* parent drugs^{30–32}. The metabolism of minoxidil is an early example of this³³. Although potent hypotensive effects are seen with the parent compound in animal models, the effects in humans are exclusively due to the sulfonated metabolite^{34,35}. More recent studies have suggested that up to 22% of the top 50 drugs that are prescribed in the US produce active metabolites that are essential to their pharmacological effects³².

3.1 Statin metabolites

Statins are widely prescribed drugs³⁶ that act to lower circulating cholesterol levels by inhibiting hepatic HMG Co-A reductase³⁷. The statins were originally derived from fungal secondary metabolites³⁸. Interestingly, several fungal CYPs are known to be involved in the statin biosynthetic pathway³⁹. One of the original statins to be developed was compactin (mevastatin), however it was never brought to market due to adverse effects in a canine animal model³⁶. The first statin approved for clinical use was lovastatin (Mevacor) from the fungus *Aspergillus terreus*⁴⁰. Since then, a series of statin analogs have been introduced, including the best-selling atorvastatin (Lipitor) and rosuvastatin (Crestor), both of which have been suggested to have active metabolites^{41–43}. Atorvastatin is predominately metabolized by CYP3A4 to form active ortho-(2-hydroxy) and para-(4-hydroxy) metabolites (Figure 3), as well as several beta-oxidation metabolites^{43–45}.

Both 2-hydroxyatorvastatin and 4-hydroxyatorvastatin exhibit plasma concentrations similar to that of atorvastatin, albeit with a somewhat reduced elimination half-life⁴⁶. In the case of rosuvastatin, it undergoes *N*-demethylation at the sulfonamide nitrogen to generate *N*-desmethylrosuvastatin, which also seems to be active, although at reduced potency⁴⁷. Interestingly, both the organic anion transporting polypeptides (OATPs) and CYPs have been demonstrated to be important determinants of the disposition of these drugs, implying that interindividual pharmacogenetic differences may play a role in statin distribution and efficacy *in vivo*^{43,47}. As new “omics” technologies become available to analyze the CYP/transporter phenotype of an individual, treatment may be able to be paired to a particular phenotype for improved response⁴⁸. Certainly, the fact that some of the metabolites

demonstrate improved pharmacokinetics over the parent compounds suggests that similar metabolites of other statins should be more fully investigated in regards to efficacy. Given that some of the CYPs involved in the fungal production of statins have been cloned and characterized for their activities, it may now be possible to produce these specific metabolites in larger quantities for further clinical study and production⁴⁹.

3.2 Antibiotics

Similar to the statins, many of the antibiotics currently in clinical use have CYPs involved at critical steps in their biosynthetic pathways^{4,50}. These include several different classes of compounds isolated from *Streptomyces* spp., such as: erythromycin, tetracycline, amphotericin B, avermectin, and adriamycin⁴. Many of the CYPs involved in these biosynthetic pathways perform tailoring functions, such as the addition of hydroxyl groups or endoperoxide bridges, near the end of the biosynthetic pathways.

The macrolide antibiotics are an important class of antibiotics in use worldwide^{4,51}. While newer analogs such as azithromycin and clarithromycin have replaced erythromycin in many countries; erythromycin is still used substantially for some indications, especially in the Third World⁵¹. Erythromycin is transformed *in vivo* into 8,9-anhydroerythromycin⁵². Although this derivative possesses no antimicrobial activity itself, it is a potent motilide – a mimic of the peptide motilin that causes duodenal contractions^{52,53}. In this case, the daughter compound has therapeutic properties that are distinct from the parent drug. In contrast, the major CYP metabolite of clarithromycin, 14-hydroxylclarithromycin (Figure 4), exhibits potent antimicrobial activity in its own right⁵⁴.

Although this metabolite has improved activity against certain *Legionella* species, its efficacy against penicillin resistant *Streptococcus pneumoniae* is lower than the parent drug, thereby demonstrating an altered spectrum of antimicrobial activity⁵⁴. Several of the bacterial CYPs in the erythromycin biosynthetic pathway have been cloned and expressed in *E. coli*, allowing for further structure-function characterization and the possibility of combinatorial biosynthesis for the production of new antibiotic derivatives, or compounds with entirely new therapeutic properties^{55,56}.

3.3 Antidepressants

Thioridazine, an older piperidine antipsychotic still used in some countries, undergoes sequential *S*-oxidation of the thiomethyl group of the thiazine ring to produce the sulfoxide (Mesoridazine) and the sulfone (Sulforidazine), each of which has been developed independently as a drug⁵⁷ (Figure 5).

Interestingly, both metabolites have higher affinity for D₂, D₃, D₄ dopaminergic and α_1 noradrenergic receptors than thioridazine itself⁵⁸, and the metabolites are less protein-bound than the parent drug, suggesting that the majority of receptor occupancy is due to the metabolites and not the parent compound⁵⁸.

The anxiolytic buspirone, a 5-HT_{1A} agonist, is metabolized both to 6-hydroxybuspirone and an *N*-dealkylated form, both of which are active⁵⁹. The 6-hydroxy metabolite is known to be relatively metabolically stable and contributes substantially to the anxiolytic activity of

buspirone⁵⁹, which led GlaxoSmithKline to attempt development of this metabolite under the name Radafaxine. This suggests that investigation of other hydroxylated versions of the parent compound may be profitable due to their improved pharmacokinetic profiles.

3.4 Antihistamines

Terfenadine, the first non-sedating antihistamine, was initially marketed in the United States in 1985 for the treatment of allergic rhinitis. However, it was withdrawn from clinical use in the late 90's due to concerns regarding arrhythmia caused by abnormal QT interval prolongation⁶⁰. This effect was exacerbated by pharmacokinetic drug interactions with azole antifungal agents, such as ketoconazole⁶⁰. Terfenadine is known to undergo extensive first pass metabolism by CYP3A4, primarily producing the carboxylic acid metabolite (fexofenadine)⁶¹. Most of the antihistaminic (H₁ receptor) effect is seen with the metabolite but fexofenadine exhibits reduced cardiac toxicity, which led to its development as a second generation non-sedating antihistamine. In this case, toxicity seen with the parent compound was avoided by an understanding of the pharmacokinetics and activity of the CYP-generated metabolite.

3.5 Muscarinic antagonists

Tolterodine is a M₂/M₃ muscarinic receptor antagonist used to treat urinary incontinence and overactive bladder⁶². It is predominately metabolized by CYP2D6 to produce 5-hydroxymethyltolterodine (desfesoterodine) (Figure 6)⁶³.

Remarkably, although both the parent and metabolite exhibit similar antimuscarinic activities, the metabolite is 10-fold less protein bound⁶⁴. Therefore, in individuals possessing CYP2D6 extensive metabolizer phenotypes, the desfesoterodine metabolite is predominantly responsible for the pharmacological effects observed^{63,64}. Conversely, in poor metabolizers, the parent compound is the major contributor. Additionally, animal studies have demonstrated that less of the metabolite than the parent drug penetrates the blood brain barrier, suggesting that the metabolite is less likely to cause off-target central nervous system effects. These data led to the development of fesoterodine, an ester prodrug of the metabolite. This example illustrates how interindividual CYP genetic variability can affect the efficacy through an active metabolite.

3.6 Antineoplastic agents

The tamoxifen CYP2D6 metabolites; norendoxifen, afimoxifene and endoxifen, were demonstrated to be potent inhibitors of CYP19A1 (aromatase) with a K_i for norendoxifen of 35 nM⁹. Extensive CYP2D6 metabolizers are thus more responsive to therapy with tamoxifen than moderate or poor metabolizers. Additionally, norendoxifen exhibited excellent selectivity for CYP19A1 inhibition when screened against a variety of drug metabolizing CYPs, including: CYP2B6, 2C9, 2C19, 2D6, and 3A4⁹. Thus, active metabolites like norendoxifen contribute directly to the anticancer effect seen with this drug and may also serve as potent and selective lead compounds upon which to build novel CYP19A1 inhibitors.

4. FUTURE DIRECTIONS AND CONCLUSIONS

One of the more innovative and exciting approaches to the identification and exploitation of active CYP metabolites has been the development of microscale and microfluidic technologies for the production and analysis of CYP metabolic products¹⁰. Using these technologies, it is possible to screen a number of CYP generated metabolites simultaneously. These new approaches will not only accelerate the pace of CYP metabolite lead discovery, but also dramatically reduce its cost. Microscale, and other, emerging technologies hold the promise of incorporating CYP metabolite lead identification into part of the drug discovery process.

CYP enzymes are found in every kingdom of life and are essential for many important biological functions, including endogenous and xenobiotic metabolism, secondary metabolite production, and organism homeostasis. In drug discovery and development, they have traditionally been considered a liability. However, the multitasked CYPs can also be a platform for drug discovery and development through the production of active metabolites, natural product lead synthesis, or even as drug targets. As new technologies emerge for metabolite production and characterization, the number of drug candidates produced from CYPs is likely to increase substantially.

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Figure 1.
Biosynthetic pathway for the anti-malarial artemisinin.

Figure 2.
Amphotericin B (1) and its analogs (2) and (3) (ref. 23).

a. Active metabolites of atorvastatin

b. Active metabolite of rosuvastatin

Figure 3.

Atorvastatin, rosuvastatin and their active metabolites.

Figure 4.
Clarithromycin and its active metabolite 14-hydroxyclearithromycin.

Figure 5.
Sequential oxidation of Thioridazine.

Figure 6.
Metabolism of tolterodine to its active metabolite, 5-hydroxymethyl tolterodine