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Four decades of CYP2B research: From protein adducts to protein structures and beyond

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Running Title

Four decades of CYP2B research

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Abbreviations

245-HCB	2,2',4,4',5'5'-hexachlorobiphenyl
ACPHS	Albany College of Pharmacy and Health Sciences
AFM	atomic force microscopy
ASPET	American Society for Pharmacology and Experimental Therapeutics
CYP1A1	cytochrome P450 1A1
CYP1A2	cytochrome P450 1A2
CYP2A6	cytochrome P450 2A6
CYP2A13	cytochrome P450 2A13
CYP2B	cytochrome P450 2B
CYP2B1	cytochrome P450 2B1
CYP2B4	cytochrome P450 2B4
CYP2B6	cytochrome P450 2B6
CYP2B11	cytochrome P450 2B11
CYP2C	cytochrome P450 2C
CYP2C3/5LVdH	chimeric cytochrome P450 2C3/5 LVdH
CYP2C5	cytochrome P450 2C5
CYP2C8	cytochrome P450 2C8
CYP2C9	cytochrome P450 2C9
CYP2E1	cytochrome P450 2E1
CYP2U1	cytochrome P450 2U1
CYP3A4	cytochrome P450 3A4

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CYP3A7	cytochrome P450 3A7
CYP3A12	cytochrome P450 3A12
CYP8B1	cytochrome P450 8B1
CYP11A1	cytochrome P450 11A1
CYP11B1	cytochrome P450 11B1
CYP11B2	cytochrome P450 11B2
CYP17A1	cytochrome P450 17A1
CYP21A2	cytochrome P450 21A2
CYP101A1	cytochrome P450 101A1
ISSX	International Society for the Study of Xenobiotics
NIEHS	National Institute of Environmental Health Sciences
MBI	mechanism-based inactivators
P-gp	P-glycoprotein
UoN	University of Nottingham
UTMB	University of Texas Medical Branch

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Abstract

This article features selected findings from the senior author and colleagues dating back to 1978 and covering approximately three-fourths of the 60 years since the discovery of cytochrome P450. Considering the vast number of P450 enzymes in this amazing superfamily and their importance for so many fields of science and medicine, including drug design and development, drug therapy, environmental health, and biotechnology, a comprehensive review of even a single topic is daunting. To make a meaningful contribution to the 50th anniversary of *Drug Metabolism and Disposition*, we trace the development of the research in a single P450 laboratory through the eyes of seven individuals with different backgrounds, perspectives, and subsequent career trajectories. All co-authors are united in their fascination for the structural basis of mammalian P450 substrate and inhibitor selectivity and using such information to improve drug design and therapy. An underlying theme is how technological advances enable scientific discoveries that were impossible and even inconceivable to prior generations. The work performed spans the continuum from 1) purification of P450 enzymes from animal tissues to purification of expressed human P450 enzymes and their site-directed mutants from bacteria; 2) inhibition, metabolism, and spectral studies to isothermal titration calorimetry, deuterium exchange mass spectrometry, and NMR; 3) homology models based on bacterial P450 X-ray crystal structures to rabbit and human P450 structures in complex with a wide variety of ligands. Our hope is that humanizing the scientific endeavor will encourage new generations of scientists to make fundamental new discoveries in the P450 field.

Key Words

Cytochrome P450 (CYP), X-ray crystallography, Nuclear Magnetic Resonance (NMR), MDR/p-glycoprotein, Snake venom

Significance Statement

The manuscript summarizes four decades of work from Dr. James Halpert's laboratory, whose investigations have shaped the cytochrome P450 field, and provides insightful perspectives of the co-authors. This work will also inspire future drug metabolism scientists to make critical new discoveries in the cytochrome P450 field.

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Introduction

As a former Editor of *Drug Metabolism and Disposition* and recipient of the Bernard B. Brodie Award in Drug Metabolism (Halpert, 2011), the senior author Dr. James Halpert was honored to be invited to prepare an article to help celebrate the 50th anniversary of the journal. To accomplish that he was fortunate to receive contributions from former members of his research group who were responsible for many of the pivotal findings on the structure and function of CYP2B enzymes over four decades. In 1978 when Dr. Halpert entered the P450 field, major unanswered questions included: How many P450 enzymes exist in the liver of different mammalian species, including humans? What substrates does each P450 act on, and by which compounds are the enzymes inhibited? What are the primary and 3D structures of the P450s, and how do the structures relate to function and inhibition? Pursuing these questions primarily through studies of CYP2B and CYP3A enzymes in multiple species was a fascinating and rewarding journey, especially thanks to the wonderful colleagues who contributed along the way. During his 40+ years studying P450s, Dr. Halpert was blessed to work with almost 100 undergraduates, graduate students, postdoctoral fellows, and visiting scientists. After an initial decade during which Dr. Halpert was still contributing significantly to data generation, virtually all his research findings from the laboratory were made by co-workers. The evolution of the research in the laboratory is therefore a story best told by the people who made the key discoveries. These stories also mirror to a considerable extent the evolution of research in the P450 field during the time frame in question. The co-authors of this article were selected to provide continuous coverage along with Dr. Halpert of the years from 1978 to the present. Although most of Dr. Halpert's former trainees actually went on to very successful careers in industry, for practical reasons it has been much easier to stay in close contact with those who

sought academic careers. That factor explains in part why only one contributor to this article is from industry. Although the article is mainly retrospective, it is gratifying to note that former lab members who remain active in research continue to push the drug metabolism field forward in many exciting new directions including P450 structure and drug design, transporter structure and function, and novel drug delivery modalities.

James Halpert

My introduction to protein biochemistry and drug metabolizing enzymes. Having become enthralled with Sweden while on an education abroad program as a junior at UCLA, I returned in fall 1971 to work in Lund. By fall 1973 I was ready to continue my education, and I joined the laboratory of another American, David Eaker, in the Department of Biochemistry at Uppsala University. The reputation of the department in protein separation science was outstanding, dating back to the Nobelists Theodor Svedberg (ultracentrifugation) and Arne Tiselius (electrophoresis). I felt extremely fortunate to be given the project of determining the amino acid sequence of a presynaptic neurotoxin from snake venom, specifically the Australian tiger snake (Halpert and Eaker, 1975). David's lab and others had determined numerous primary structures of post-synaptic (curarimimetic) toxins, but no presynaptic toxin had been sequenced, and the mechanism of action was unclear. The notexin sequence and functional assays clearly demonstrated that it was a phospholipase A2, and chemical modification of a single His-48 residue showed the importance of the enzymatic activity for neurotoxicity (Halpert *et al.*, 1976). Two other related projects fleshed out my Ph.D. dissertation, and I became interested in migrating from toxinology to toxicology by enrolling in a new M.S. Program in Toxicology at the Karolinska Institute.

There I met I Magnus Ingelman-Sundberg, who became a life-long friend. My thesis with Magnus was on the purification and characterization of rabbit liver epoxide hydrolase (Halpert *et al.*, 1979). I also encountered cytochrome P450 for the first time as a pale-yellow contaminant in the penultimate epoxide hydrolase preparation. It did not dawn on me that I would spend much of the next 15 years purifying P450 enzymes as opposed to trying to remove them from preparations of other enzymes.

The project that launched my independent career. With a Ph.D. in Biochemistry and M.S. in Toxicology, I decided to return to the U.S. in fall 1978. The perfect place to marry those two interests was the Center for Environmental Toxicology at Vanderbilt University, which was directed by Robert (Bob) A. Neal in the Department of Biochemistry. I was inspired by Bob's wisdom, the excitement for science of my lab mates, and the dynamism of our neighbor Fred Guengerich. My career plans were vague, but I had learned in Uppsala how valuable it could be to tackle a problem that had not been solved before but was nonetheless tractable. Fortunately, such a problem came along.

My project was to use mechanism-based inactivators (MBIs) to label P450 active sites. Most P450 MBIs known at the time labeled or destroyed the heme moiety of the enzyme, thus providing no information about active site residues. After I completed a project on inactivation of cytochrome P450 2B1 (CYP2B1) by parathion, another postdoctoral fellow, Chris Chengelis, showed me an article suggesting that the antibiotic chloramphenicol might be more suitable. Indeed, CYP2B1 was inactivated by chloramphenicol with no loss of P450 or heme, and the protein was labeled almost stoichiometrically. That finding set the stage for identification of *N*-ε-chloramphenicol oxamyl lysine (Halpert, 1981) as the adduct comprising about half of the bound chloramphenicol (Figure 1). Key steps were: 1) elaboration of conditions for enzymatic digestion

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of CYP2B1 labeled with [^{14}C]chloramphenicol in a reconstituted system or liver microsomes; 2) chemical and chromatographic characterization of a hydroxylamine stable adduct that released chloramphenicol acid upon treatment with 1 N NaOH; 3) use of microsomes to generate sufficient material for purification, including a final step employing the ion exchange column on a commercial amino acid analyzer; 4) comparison with the synthetic adduct as a standard. Later, after returning to Sweden as a research track faculty member in Jan-Åke Gustafsson's department, I demonstrated the formation of the lysine adduct of chloramphenicol and CYP2B1 *in vivo* (Halpert *et al.*, 1983). These findings represented the first identification of an amino acid adduct of a reactive intermediate of a xenobiotic with a P450 enzyme. Although such adduct identification might be straightforward now using modern mass spectrometry of proteins, the work helped me stand out in competition for a tenure-track faculty position. Furthermore, 30 years after publication of my work on the lysine adduct of chloramphenicol and CYP2B1, Sean Gay in my group in collaboration with Haoming Zhang and Paul Hollenberg at the University of Michigan was able to solve two crystal structures of CYP2B4 covalently modified by a metabolite of the MBI metabolite *t*-butyl phenylacetylene (reviewed in Halpert, 2011). That work was completed more quickly than my work at Vanderbilt on a single chloramphenicol adduct of CYP2B1.

Early years as a faculty member. I was fortunate to be the first assistant professor hired by a new department head, Glenn Sipes, in the Department of Pharmacology and Toxicology at the University of Arizona. At the time, biotransformation was the core of the interdisciplinary toxicology program, and multiple faculty members and graduate students were interested in my research. My main challenge was how to carve out a niche for myself in what was then the extremely competitive field of cytochrome P450. Thanks to a fortuitous discussion with Dick

Philpot from the National Institute of Environmental Health Sciences (NIEHS), I realized that working at the interface between pharmacology and biochemistry was the key. Although the availability of selective inhibitors of the major human P450 enzymes involved in drug metabolism is almost taken for granted today, at the start of my independent career very little was known about the selectivity of any P450 inhibitor in any species. Thus, my path forward was to first characterize and then enhance the selectivity of chemical inhibitors towards the rat and rabbit P450 enzymes known at the time. Pursuing that problem was the start of an independent research career that led me into so many other aspects of P450 structure-function and structure-activity relationships involving enzyme purification, inhibitor design, site-directed mutagenesis, homology modeling, X-ray crystallography, and solution biophysics.

For the first six years of my faculty position, my research group consisted mainly of graduate students pursuing projects reliant on my own expertise in protein biochemistry. I will briefly mention three students whose work had long-term implications for the research in our group. Dave Duignan was the first to purify a dog liver cytochrome P450, later termed cytochrome P450 2B11 (CYP2B11). His Ph.D. studies showed the importance of this enzyme for the unique ability of dogs to metabolize and eliminate certain polychlorinated biphenyls, including 2,2',4,4', 5'5'-hexachlorobiphenyl (245-HCB) (Duignan *et al.*, 1987). This was the pivotal first step in three subsequent decades of structure-function studies of cytochrome P450 2B (CYP2B) enzymes in multiple species. Paul Ciaccio purified the second dog liver P450, named cytochrome P450 3A12 (CYP3A12) (Ciaccio and Halpert, 1989). His work eventually led to two decades of research on cooperativity of cytochrome P450 3A4 (CYP3A4). Jeff Stevens did both his M.Sc. and Ph.D. with me on MBI, and his experiences are described below.

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Transition to cDNA cloning and heterologous expression. By 1989, I realized the need to learn basic recombinant DNA technology if my laboratory was going to survive. After returning from a sabbatical at UCLA with some knowledge and competence in recombinant DNA, I was able to recruit very talented postdoctoral fellows who pushed our research in multiple directions far beyond my own expertise. We stopped working with purified proteins from animal tissues and relied totally on CYP2B enzymes expressed in yeast, mammalian cells, and eventually bacteria. This transition enabled a very productive decade of site-directed mutagenesis studies to predict the CYP2B active site and to explain functional differences among CYP2B enzymes from rats, rabbits, and dogs. The prerequisite for success was using substrate metabolite profiles as opposed to rates of overall turnover as the functional marker. This approach (Domanski and Halpert, 2001) allowed us to predict virtually all of the CYP2B active site residues subsequently identified by X-ray crystallography (Shah *et al.*, 2012, 2015). The experiments were complemented by homology models, including the first one to use three rather than a single bacterial P450 X-ray crystal structure and experimental results to help refine the sequence alignments (Szklarz *et al.*, 1995).

Jeffrey C. Stevens

From undergraduate to The University of Arizona. My path from my time as an undergraduate at the University of Notre Dame to graduate school at the University of Arizona was characterized by some planning, but much more by angst and serendipity. While many of my friends had clear post-graduate plans by the end of their junior year, I was puzzled. Ideas came into focus, however, when I started an undergraduate research project with Dr. Ken Olson, a professor in Physiology at the Indiana University School of Medicine. Ken was brilliant but humble and had a true passion for science and working with his students. I believe that the two

semesters of sporadic work in Ken's lab gained me all of two credits toward graduation, but much more importantly focused me on finding a graduate program where I could study some combination of biology and chemistry. I managed to jump that hurdle when I was accepted into the Master's Program in Toxicology at the University of Arizona in 1985. I arrived in Tucson elated to start the new adventure but largely naïve to the discipline of toxicology. One of the first, and in retrospect, career determining tasks, was to select a research advisor. Over the past 37 years, Jim Halpert and I have frequently recalled our first interaction. I was standing in the hall outside of Jim's lab, reading some of the reprints of his work posted on the bulletin board. He approached me, introduced himself, and gave me what is now referred to as an 'elevator pitch' on his research and lab group. Over the next few days, Jim hosted me for long meetings in his office where he enthusiastically summarized the field of cytochrome P450 and expounded on the future research opportunities to study drug metabolism from the perspective of this quirky enzyme superfamily. With a handshake, a lab notebook, and a nudge toward the lab I embarked on my career association with Jim.

For perspective, in the mid-1980s P450 research was just emerging from the often-heated debates as to whether there were multiple forms of the enzyme. Furthermore, purification of highly related P450s was a labor-intensive art practiced by only a handful of investigators. Therefore, differentiating various rat P450s by a combination of selective enzyme activity and chemical inhibitors was a plausible means of studying the P450s found in rat liver microsomes. The work of David Waxman at Harvard (Waxman *et al.*, 1983) and Fred Guengerich at Vanderbilt (Waxman *et al.*, 1985) on the P450-selective hydroxylation of various endogenous steroids provided the starting point for my Master's research project. Jim showed me the few simple steps for the synthesis of chloramphenicol analogs, and eventually, the NMR and

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elemental analysis of the products confirmed my first (and only) success as a medicinal chemist. Whether I was performing enzyme assays, tinkering with affinity chromatography columns while purifying P450 reductase, or talking science with the other graduate students, time seemed to fly when I was in the lab. The result of the work was my first publication (Stevens and Halpert, 1988), and I have always retained the acceptance letter from the editor of *Molecular Pharmacology*, Dr. Bill Catterall.

Ph.D. Dissertation. With the support from my family, I continued to the Ph.D. program in Pharmacology and Toxicology at Arizona in Jim's lab. I was encouraged to become more independent, and my project shifted to studying mechanism-based inactivators of adrenal P450s. The cow was the obvious source for obtaining the large amounts of tissue needed for studying the P450 C-21 (CYP21A2) and 17-α (CYP17A1) enzymes. The problem was that commercially available frozen cow adrenal glands yielded very low enzyme activity. So, one day I packed up a Styrofoam cooler and, armed with a large knife, headed east from Tucson to find a local slaughterhouse. The trip was successful, but for this city kid, the slaughterhouse experience was an extreme shock of sight and smell. However, the science advanced, and we proved that 17β-substituted steroids were efficient inactivators of bovine adrenal CYP21A2 and CYP17A1. Fig. 2 from our 1991 publication in *Biochemistry* (Stevens *et al.*, 1991) exemplifies the breadth of my graduate research efforts and shows the spectrum of the purified CYP21A2 after irreversible binding of a reactive metabolite of a steroid analog.

Postdoctoral training and career advancement. Jim held a high standard for writing and external presentations, and seminar practice sessions usually extended well past the allotted time. However, the invested effort paid off, starting with my first interview for a postdoctoral scientist position at Eli Lilly and Company. My hiring manager, Dr. Steve Wrighton, secretly confiscated

my painstakingly prepared seminar script just minutes before I was introduced. That was just the beginning of thinking on my feet during my 24-year journey as a scientist and senior manager in the pharmaceutical industry. During my first job as a Senior Scientist at Rhone-Poulenc Rorer, the application of P450 research to human drug metabolism during drug discovery exploded. Screening drug candidates for P450 inhibition was required, and understanding the broad substrate affinity of the seemingly ubiquitous CYP3A4 resurrected many of the questions from Jim's continuing work on P450 structure and function. When I moved on to a position at Pharmacia, I was fortunate to be encouraged constantly by Dr. Larry Wienkers to collaborate with leading academic labs, hire postdocs, publish, and increase my Editorial Board responsibilities with ASPET Journals. His emphasis on internal and external professional growth served as an example that I tried to emulate as I moved up to Senior Director at Pfizer in 2004. After eleven years of working on an amazing drug pipeline with many scientists I now call friends, I moved on to work with industry as an independent consultant. Due to my passion for and continued involvement with ASPET Journals, I had the unique opportunity to be named the first editor of *Drug Metabolism and Disposition* to have had a career in the pharmaceutical industry. Not coincidentally, the first person I informed of the news of this honor was none-other than a former DMD editor and friend, Jim Halpert.

Grazyna D. Szklarz

From fungal peroxidases to mammalian cytochromes P450. My research career started in Poland, where I obtained an M.Sc. degree in Biology with a major in Molecular Biology. I worked on degradation of lignin by wood-rotting fungi and the role of various oxidative enzymes in this process. I then continued to work in this field during my Ph.D. research at Clarkson University, Potsdam, NY, where my focus was on fungal oxidases and peroxidases. After

graduating with a Ph.D. in Chemistry, major in Biochemistry, I looked for a postdoctoral position in a related area of research. My graduate education and research gave me an excellent background in biochemistry and enzymology, as well as in molecular biology and physical chemistry, which was of great help in my future career. I was always interested in how biological systems worked, in particular enzymes and other important macromolecules. Since an early age, I wanted to pursue a career in science, influenced perhaps by the example of Marie Curie, who was my personal heroine, highly respected in Poland.

I switched from the field of lignin degradation to cytochromes P450 starting with my first postdoctoral position, which was at UCSF in the laboratory of Walter Miller. I was drawn to work on P450s due to their importance in human health, and my knowledge of other heme enzymes, such as fungal peroxidases, made the transition easier. Walter's lab was focused on steroidogenic P450 enzymes, and my work there involved the function of P450scc (CYP11A1), responsible for side-chain cleavage of cholesterol (Black *et al.*, 1994).

Molecular modeling of cytochromes P450. My second postdoc, which was to be of crucial importance for my scientific career, was in the laboratory of Jim Halpert, then at the University of Arizona in Tucson. It was there that I first learned and applied molecular modeling methods to study structure-function relationships of cytochrome P450 enzymes. The time was right for the then-new modeling methodology, as only one P450 crystal structure was known, namely that of bacterial P450cam (CYP101A1). The initial steps into this area were quite modest: sequence alignment and display of key residues using the CYP101A1 structure, as described (Hasler *et al.*, 1994). Learning proper molecular modeling techniques required some time and effort, and there were relatively few researchers who worked in this area. Fortunately, Jim sent me to San Diego for a short course in molecular modeling at Biosym (now Accelrys), which was crucial for my

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future modeling efforts. We also established collaboration with Rick Ornstein, a leader in the molecular modeling field, then at Pacific Northwest National Laboratory, Richland, WA.

Our first homology model, that of CYP2B1, was based on the structure of P450cam (Szklarz *et al.*, 1994) and was used to interpret the results of site-directed mutagenesis experiments, thereby linking modeling methods with experimental results. This model also suggested that additional amino acid residues may be important for enzymatic activity. Therefore, we made several other CYP2B1 mutants, assessed their activities, and constructed an improved enzyme model based on the three bacterial structures then available (Szklarz *et al.*, 1995). This new model helped explain changes in activity upon residue substitution at key amino acid positions. Fig. 3A shows the effect of replacement of Ile-209 with Ala in CYP2B1, which allows for binding of progesterone in a new orientation, consistent with the formation of the new metabolite 21-OH progesterone. In addition to modeling and enzyme assays, stoichiometry of substrate oxidation was employed to probe the active site of CYP2B1 using 7-alkoxycoumarins as substrates (Kobayashi *et al.*, 1998). A combination of site-directed mutagenesis, construction of hybrid enzymes, enzyme assays and molecular modeling was also successfully used to identify and explain the role of key residues in rabbit CYP2B4 and 2B5 (Szklarz *et al.*, 1996; YQ He *et al.*, 1996). A similar approach was also applied to study human CYP3A4, starting with a homology model of this enzyme (Szklarz and Halpert, 1997).

As I acquired more expertise in homology modeling and docking methods, we participated in various collaborative projects, in particular with Almira Correia from UCSF (K He *et al.*, 1996; Wang *et al.*, 1998) and Paul Hollenberg from the University of Michigan (Kent *et al.*, 1997). Several of those projects involved providing a rationale for enzyme inhibition and/or inactivation. For example, Figure 3B shows BBT (*N*-benzyl-1-aminobenzotriazole) docked in the active site

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of CYP2B1 in an orientation allowing for oxidation at the 1-amino nitrogen. When Gly-478 is replaced with Ala (green), steric overlaps prevent oxidation at this position, and thereby enzyme inactivation. I really enjoyed working on those collaborative projects – they were very stimulating and required a thorough understanding of mechanisms of diverse reactions; figuring this out was part of the fun!

Looking back, working in Jim's lab was one of the best and most productive (17 publications) times in my research career. I am very grateful for his guidance and leadership. In addition to learning many laboratory techniques, I also gained some practice in grant writing, which proved handy when I started my independent career. We became really good colleagues and friends.

More P450s, but now independently. When I became an Assistant Professor at West Virginia University, I chose to study human CYP1A1 and 1A2, mainly due to their involvement in cancer. I was able to obtain a couple of R15 grants from NIH, and I was also an investigator on our Cancer Center COBRE grant. My lab continued to use previously established modeling and experimental methods to study our chosen enzymes. Moreover, we also expanded our molecular modeling methodology, applying those techniques for determination of binding constants (Szklarz and Paulsen, 2002; Liu *et al.*, 2003), and prediction of product profiles (Ericksen and Szklarz, 2005; Tu *et al.*, 2008). We had some collaborative projects as well that utilized our modeling expertise to study various P450 enzymes, including CYP1A1, which was crystallized by Emily Scott (Walsh *et al.*, 2013a), CYP2C9 (Rademacher *et al.*, 2012) and CYP 2B enzymes (Huo *et al.*, 2017). More recently, we have successfully used NMR methodology to study the function of CYP1A1 and 1A2 (e.g., Huang and Szklarz, 2018).

On the whole, I have used molecular modeling techniques extensively in my P450 research, starting with the first homology model in Jim Halpert's lab. Today, many P450 X-ray crystal

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structures are known, and other physical biochemistry methods such as NMR, are more readily available. However, there is still a need to use molecular modeling, especially molecular dynamics, to explain certain aspects of P450 function, such as interactions with redox partners and the lipid membrane.

Emily E. Scott

Introduction to heme proteins. My interest in heme proteins started with starfish. During a Marine Biology B.S. at Texas A&M University at Galveston, my big break came in a late-night phone call offering a last-minute spot on a multi-week research expedition. On such offshore trips, the key discovery was generally some odd brittle stars, the only living thing in severely oxygen-depleted waters. Mostly buried in sediment, their waving arms were easily visible because of their red water vascular system. This color derived from the unusual presence of hemoglobin. Having learned about myoglobin and hemoglobin in the early chapters of every biochemistry textbook, I wondered what gave brittle star hemoglobin high enough oxygen affinity allowing survival? I was intrigued ever after by heme proteins.

While I enjoyed diving and fieldwork immensely, I was better suited to experiments with tighter control of experimental parameters. Thus, I did my Ph.D. in the myoglobin/hemoglobin laboratory of John S. Olson in the Biochemistry and Cell Biology Department at Rice University in Houston. Here I learned recombinant heme protein expression (100 L fermentor!) and purification, unfolding (Scott *et al.*, 2000), and pre-steady state ligand binding using stopped-flow and laser flash photolysis (Scott and Gibson, 1997; Scott, Gibson, *et al.*, 2001). I had the good fortune to also work with Quentin Gibson, developer of the stopped-flow and a consummate British gentleman. I was fascinated by heme protein structure/function relationships but wanted an enzyme.

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Becoming entranced by membrane P450 structure in the Halpert lab. Cytochrome P450 literature excited me, particularly the potential for environmental cleanup, which appealed to my environmentalist background. For a postdoc I applied to P450 labs including that of James (Jim) Halpert, then at University of Texas Medical Branch (UTMB). The project that sparked my enthusiasm was that of P450 structure determination. There were a few soluble P450 structures, but none for membrane/mammalian enzymes. My first crystallization experiments consumed the modest amount of CYP2B1 Jim had arduously isolated from phenobarbital-induced rats. After working with grams of recombinant myoglobin I hadn't appreciated how hard-won that partial vial of CYP2B1 was. So I first needed to generate significant quantities of protein. Engineering CYP2B enzymes from multiple species (Scott, Spatzenegger, *et al.*, 2001) followed the N-terminal modifications Eric Johnson's lab used for cytochrome P450 2C (CYP2C) (Cosme and Johnson, 2000), yielding enough protein to restart crystallization. Jim arranged for me to visit Eric's Scripps lab to learn from their success determining the first mammalian structure, chimeric cytochrome P450 2C3/5 LVdH (CYP2C3/5LVdH) (Williams *et al.*, 2000). However, before then, I obtained CYP2B4 crystals and collected diffraction data, so I took the data to analyze under the guidance of Eric's crystallographic collaborator, David Stout. The first day of solving this structure I'd become entranced by the puzzle-like beauty of atomic arrangements. Refinement revealed that cytochrome P450 2B4 (CYP2B4) had a very wide active site cleft (Scott *et al.*, 2003), never seen before in soluble P450 enzymes or Eric's CYP2C structure. We didn't know if this was typical for CYP2B4 overall or a conformational state (Fig. 4). A subsequent liganded structure (Scott *et al.*, 2004)—generated after sleep-deprived trips to the synchrotron—indicated the latter and allowed us to envision P450 opening and closing to

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accommodate ligand entry/exit. I was transfixed by the fascinating variety of P450 structures (Fig. 4).

Starting off on my own, but with P450 friends. I started my laboratory in the University of Kansas Medicinal Chemistry Department focusing on the structural biology of drug-metabolizing human P450 enzymes. We were the only biochemistry lab, but I had chairs who mostly left us to our own devices and early support of a COBRE grant run by Robert (Bob) Hanzlik. We fortunately obtained cytochrome P450 2A13 (CYP2A13) crystals (Smith *et al.*, 2007) and an NIH grant the first year. Over time multiple structures of cytochrome P450 2A6 (CYP2A6) (DeVore *et al.*, 2008, 2012; DeVore and Scott, 2012a), cytochrome P450 2E1 (CYP2E1) (Porubsky *et al.*, 2008, 2010), and cytochrome P450 1A1 (CYP1A1) (Walsh *et al.*, 2013b) followed, each a hard-won, multi-year triumph. This work led to the Early Career (now Richard Okita) Award from American Society for Pharmacology and Experimental Therapeutics (ASPET) and the International Society for the Study of Xenobiotics (ISSX) North American New Investigator Award in honor of James Gillette, but more important was developing connections with wonderful colleagues in the P450/drug metabolism fields.

Seeking new challenges, I read that inhibitors were being developed for steroidogenic cytochrome P450 17A1 (CYP17A1) to treat prostate cancer. I enlisted Jeff Aubé, a synthetic chemist colleague, to make the abiraterone molecule then in clinical trials. With this we determined the first CYP17A1 structure (DeVore and Scott, 2012b), followed shortly by those with all four major substrates (Petrunak *et al.*, 2014) and non-steroidal inhibitors (Petrunak *et al.*, 2017). This work countered a long-standing proposal that CYP17A1 had two active site lobes for its hydroxylase and lyase functions and revealed a stronger emphasis on substrate-based chemistry. Like many in the P450 field, our research was championed by Dick Okita at NIGMS.

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This resulted in an NIH MERIT award, which continued to support work on P450 drug targets as my lab moved to the University of Michigan. Recent successes include cortisol-generating cytochrome P450 11B1 (CYP11B1) (Brixius-Anderko and Scott, 2019), aldosterone-producing cytochrome P450 11B2 (CYP11B2) (Brixius-Anderko and Scott, 2021), bile-acid-generating cytochrome P450 8B1 (CYP8B1) (Liu *et al.*, 2022), and fetal cytochrome P450 3A7 (CYP3A7) (collaboration with Jed Lampe, University of Colorado).

From CYP2B4 on, it was apparent that flexibility is a key aspect of P450 functionality. It seemed to me that NMR was an excellent complementary technique for probing P450 dynamics and transient interactions with redox partners. Using NMR, we have been able to identify structural differences among drug-metabolizing P450 enzymes (Bart and Scott, 2017) and effects on CYP17A1 structure (Estrada *et al.*, 2013, 2014, 2016) upon interaction with reductase or cytochrome b_5. A long-standing and ongoing effort is to determine a human P450 structure by NMR, now a collaboration with Tom Pochapsky (Brandeis University), who has done so successfully with soluble $P450_{cam}$.

I am grateful for excellent mentors and P450 friends. At different stages there has always been the right person to train, encourage, collaborate, and muse about the mysteries of P450s. With such mentorship comes the opportunity and responsibility to invest in the next generation. While all but one of my mentors and collaborators have been men, most of the trainees in my lab are women, and supporting a more diverse generation of scientists is a challenge for all, equally important to enable future progress in the P450/drug metabolism field.

Santosh Kumar

Research background before entering the cytochrome P450 field. My graduate thesis was on "γ-aminobutyric acid (GABA) metabolism in *Aspergillus niger*." In this study, I examined

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GABA metabolism, a shunt pathway of the citric acid cycle, in *A. niger,* a fungus known for citric acid fermentation (Kumar, Punekar, *et al.*, 2000). In this project, I also purified and characterized an important GABA pathway enzyme: succinic semialdehyde dehydrogenase (Kumar *et al.*, 2015). As a postdoctoral fellow, I studied the role of the dynein molecular motor in vesicle transport in *Neurospora crassa*, a model organism used to study both genetics and biochemistry of fungi. In this study, I purified and characterized dynein and its activator, dynactin, in *N. crassa* (Kumar, Lee, *et al.*, 2000; Kumar *et al.*, 2001).

My ambition was to become an independent researcher in academia, where I would have the freedom to work on research that I am passionate about. Research on CYP enzymes had immediate public health interest and a relatively high chance of funding compared to fungal biochemistry. In addition, my ambition was to become a good teacher and mentor as well as an academic leader in research. My journey with Dr. Halpert began with a one-on-one interview at the Experimental Biology 2001 meeting in Orlando, FL. In that interview, Dr. Halpert stated, "If you have succeeded in publishing high-quality work as a graduate student and postdoctoral fellow in two different areas of research, I believe that you can succeed in cytochrome P450 (CYP) research as well." It was this belief that gave me the confidence to pursue my research career in CYP enzymes.

One major project I accomplished that helped set the stage for my future career. I worked with Dr. Halpert from 2001-2008 as non-tenure track faculty member and was involved in multiple projects related to structure-function relationships of CYP enzymes. One project, in which we engineered CYP2B1 to behave like cytochrome P450 2C5 (CYP2C5) by mutagenesis of active site residues (Kumar *et al.*, 2003), gave me confidence to initiate a project on the engineering of mammalian CYP enzymes by directed evolution. In a strategic move, Dr. Halpert

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enlisted the help of Dr. Frances Arnold, a pioneer in bacterial CYP engineering using directed evolution and later recipient of the Nobel Prize in Chemistry in 2018. With Dr. Arnold's technical and intellectual support, we developed and implemented a directed evolution approach to engineer a more efficient mutant of CYP2B1 (Kumar *et al.*, 2005) (Fig. 5). Specifically quadruple mutations at the non-active site residues (V183L/F202L/L209A/S334P) yielded enhanced enzyme efficiency (k_{cat}/K_m) for the metabolism of model substrates (Kumar *et al.*, 2005). Interestingly, in collaboration with Dr. David Waxman from Boston University, we found that some of these mutants also exhibited enhanced efficiency with anti-cancer prodrugs, cyclophosphamide (CYP2B1 L209A/S334P) and ifosfamide (CYP2B1 V183L/L209A). Based on this project, we also reengineered CYP2B11 for enhanced enzyme efficiency with cyclophosphamide and ifosfamide (Sun *et al.*, 2007).

Dr. Waxman's goal was to identify CYP enzymes or their variants, which can efficiently activate cyclophosphamide and/or ifosfamide prodrugs into therapeutically active drugs with minimal side effects and kill cancer cells using bystander techniques. These CYP mutants, obtained using a directed evolution approach, were filed for a provisional patent. Unfortunately, the mutants did not show a sufficient improvement in efficacy and decreased toxicity in animal models to warrant further study. However, the project provided a proof-of-principle that the directed evolution approach can be used to engineer mammalian CYP enzymes for improved functions. In addition, it was invaluable for my career development that I was entrusted completely to manage the collaboration with Dr. Waxman.

Independent research career and accomplishments. My research ideas for an independent academic career were initiated when Dr. Halpert and I had lunch together in a San Diego restaurant, and he encouraged me to find a research question different from the main theme in his

group but that utilized my research training on CYP enzymes. The idea appealed to me because earlier, I had changed my research topics twice while building on the same research training. Eventually, I found my niche in studying the role of CYP pathways in the mediation of HIV pathogenesis by drugs of abuse. Since the early 2000s, induction and inhibition of CYP enzymes by anti-HIV drugs have been known to impact many anti-HIV therapies. Similarly, CYP enzymes were also known to interact with many drugs of abuse, including alcohol and tobacco. Furthermore, the literature suggested that alcohol and tobacco are 2-3 times more prevalent in the HIV population than in the general population and are known to exacerbate HIV pathogenesis. Therefore, in 2009, I initiated two independent projects, one each on alcohol and tobacco and their roles in HIV pathogenesis. In the past 13 years, our group pursued these projects extensively and published >90 peer-reviewed papers.

Working together with Dr. Halpert also sowed a seed of leadership in me, and I developed an interest in academic and scientific leadership in 2016. However, it required significant training and a change in mindset because I am not a natural leader. Therefore, I sought training from the Academic Leadership Fellows Program of the American Association of College of Pharmacy in 2017-18. A subsequent discussion with Dr. Halpert helped me focus my leadership goals. Eventually, I took a position as Assistant Dean of Scholarly Integration and Collaboration at the College of Pharmacy, University of Tennessee Health Science in 2019, which has been very rewarding and encouraged me to keep my eyes open for other opportunities. I also took a leadership role in the Society on NeuroImmune Pharmacology as Secretary followed by President of the society. As I learned from Dr. Halpert, I always try to “Give credit for success to the constituents we serve and take responsibility for our collective failure.”

In summary, Dr. Halpert not only mentored me to become an independent researcher and educator but also inspired me to lead an academic program and scientific society. I am thankful that he continues to take an interest in my success as a scientist, educator, and academic leader and to support my growth in these areas.

Arthur G. Roberts

Graduate Work at Washington State University. My scientific journey can be considered a tale of four fields. My scientific journey is probably an atypical one and one I would not believe myself if I had not lived it. I was an awful student, but I thrived in graduate school at Washington State University because grades did not matter. I worked in a photosynthesis laboratory, which was something to behold. One might expect such a laboratory in a post-apocalyptic world that swallows up the meek. The photosynthesis lab was perpetually enveloped in darkness, creating a depressive and moody atmosphere. The only light to occasionally breach the darkness came from a powerful laser in one of the rooms. Discarded stepper motors, instrument parts, and half-assembled computers lay strewn everywhere, creating the impression of a futuristic junkyard. In another room, the smell of ozone often permeated the air due to the arcing voltage from one of the roughly fashioned instruments.

Traversing the laboratory was almost like crossing a minefield where touching any dangling wire could be your last. My lab mates and I formed a motley crew of graduate students that fit well into these feral surroundings where only the strong survive. This laboratory was run by an eccentric genius named Dr. David Kramer, whom we referred to casually as “Dave.” He roughly resembled Kramer from Seinfeld and could imitate him perfectly. As a graduate student, I remember Dave rushing in and out of the lab like a possessed mad scientist. He would work on projects that most scientists considered impossible. His sheer intelligence was intimidating to

even the best scientists. Dave's genius and most significant gift to me were his creativity, limitless imagination, and scientific intrepidation. Under Dave's guidance, I authored almost a dozen manuscripts, including one on critical domain movements involved in photosynthetic electron transport (Fig. 6A, Roberts *et al.*, 2002). Most importantly, this training gave me the confidence and requisite knowledge to enter any scientific field I desired.

Postdoctoral training at the University of Washington. After graduate school, I transitioned to the cytochrome P450 (P450) field at the University of Washington Medical School. The P450 laboratory was considerably cleaner and more modern than the laboratory that I left. My new laboratory had a central pathway flanked by cubicles colored different shades of beige, once popular in the 1980s. Experiments were performed on instruments located outside these cubicles and in adjacent rooms. The stark differences from my previous laboratory required some adaptation. Accustomed to working in darkness, I often felt like I should be shielding my eyes and cowering underneath the blinding overhead lights in the laboratory. In addition, the instruments felt strange and unnatural to me. They no longer revealed their inner workings or crackled from arcing voltage. Instead, the internal components were safely ensconced within heavy beige metal boxes with digital readouts on the outside. My lab mates were much more civilized than before and wore sterile white laboratory coats that served as de facto uniforms. Adjusting to the new laboratory was slow, but I eventually adapted to the new environment.

Bill Atkins ran the laboratory. His casual dress and demeanor reminded me of a charismatic jock from a typical high school. His calming presence functioned as a sedative for much of the laboratory. However, I felt a natural instinct because of my feral upbringing to disrupt the calmness by eliciting a rise out of Bill. My first success was purifying isotopically-labeled CYP3A4 within a week. Encouraged by that, I provoked the most significant reaction in Bill by

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casually slapping down a finished CYP3A4 manuscript on his desk after only a few months (Fig. 6B, Roberts *et al.*, 2005). I had a scientific interest in investigating P450s by NMR spectroscopy, but I wanted to do something relatively unique. I envisioned using NMR to efficiently determine drug-bound P450 structures. Achieving this vision required training in advanced NMR theory, linear algebra, and molecular dynamics. My efforts culminated in an NMR-derived structure that identified drug-drug interactions within the CYP3A4 X-ray crystal structure (Roberts, Yang, *et al.*, 2011). Unfortunately, by the start of the Great Recession of 2008, faculty and some industry interviews had not gone as well as hoped. I was bloodied but not deterred. I was going to be a professor, but I needed more training. Unfortunately, time and money were running out, and I needed to find a new research position fast.

Research scientist at U.C. San Diego. Where was I going to receive the training that I needed to be a professor? At the most opportune time, I received a call from Jim Halpert asking me to join his research group at U.C. San Diego. Jim was going to help me become an independent investigator and professor. In exchange, I was going to provide my expertise and knowledge in building drug-bound P450 structures by NMR for his laboratory. This collaboration would complement Jim's X-ray crystallography work with drug-bound P450 structures. Together, we published parallel NMR-derived and X-ray crystal structures with antiplatelet drugs and cytochrome P450 2B4 (CYP2B4) (Gay, Roberts, *et al.*, 2010). Using my NMR approaches, we also determined the structures in the sequential metabolism of the analgesic amidopyrine by CYP2B4 (Roberts, Sjogren, *et al.*, 2011). We had regular discussions on the fine art of interviewing and research. Because the P450 field was mature and attaining grant funding would be challenging, we discussed other viable research areas. A P-glycoprotein (P-gp) seminar by Dr. Geoffrey Chang gave me ideas for a possible research area outside of P450s. A slide during the

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seminar showed that P-gp could withstand an unusually high number of amino acid substitutions without losing its function. Instantly, I knew I could exploit this characteristic with my NMR approach, so transitioning to the P-gp field became possible. Jim was very encouraging, and I remember him telling me that I was ready to go out and claim my faculty position. With that advice and despite some trepidation, I ventured out on faculty interviews with renewed confidence and ultimately was accepted as a faculty member of the University of Georgia.

Faculty position at the University of Georgia. Starting a professorship in a new field is a harrowing experience. The P-gp field presented numerous barriers to overcome and many failures to surmount. Regardless of the obstacles or the challenges, I always kept moving forward. In 2016, I published my first P-gp paper (Fig. 6C, Ledwitch, Barnes, *et al.*, 2016). Soon afterward, I successfully used my NMR approaches to identify drug functional groups interacting with the transporter (Ledwitch, Gibbs, *et al.*, 2016). A couple of years later, we used atomic force microscopy (AFM) to investigate the dynamics of individual P-gp transporter molecules in solution (Sigdel *et al.*, 2018). Thus, inspired by a single slide from a seminar and the additional training from Jim Halpert, I developed a viable research program and obtained roughly $3 million in NIH and other funding. Furthermore, my skills, knowledge, and training have recently allowed me to enter the targeted drug delivery field (Fig. 6D). Transitioning between four scientific research fields was quite an adventure.

Manish Shah

My introduction to protein biochemistry and drug-metabolizing enzymes: After I obtained my B. Pharm. degree from India, my initial goal was to pursue higher studies in pharmaceutical sciences and then a career in industry. However, I was intrigued by the MSc program in Applied Biomolecular Technology at the University of Nottingham (UoN) in the U.K. It was a new

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beginning coupled with the challenges of studying and navigating life abroad, which I had never thought about a few months earlier. The program included varied courses such as drug delivery, antibody biotechnology, and macromolecular hydrodynamics, and the highlight was the possibility of a summer internship at a pharmaceutical company. My internship at AstraZeneca at Alderley Park on protein refolding in the protein sciences department changed the course of my research interests from formulations to protein sciences. I decided to pursue a Ph.D. in protein biochemistry and joined the laboratory of Malcolm F. G. Stevens, known for the development of temozolomide, and worked with my advisors Drs. Charles Laughton, Andrew Westwell, and Tracey Bradshaw within the School of Pharmacy at UoN. My research included the expression and purification of recombinant mycobacterium tuberculosis thioredoxin and thioredoxin reductase from *E.coli* and determining the ability of new quinol molecules to inhibit the bacterial redox system (Hall *et al.*, 2006; Shah *et al.*, 2006). The excitement of crystallizing thioredoxin led me to seek a postdoctoral position in structural biology. I joined Dr. Andrew M. Gulick's laboratory at the Hauptman-Woodward Institute in Buffalo, where I learned the nuts and bolts of molecular biology, protein biochemistry, and X-ray crystallography. This training enabled my subsequent success in the expression, purification, and crystallization of various CYP enzymes.

I continued my postdoctoral research in the laboratory of Dr. James R. Halpert at U.C. San Diego, studying the structure-function relationships of CYP2B enzymes. At that time, no structures were available for human cytochrome P450 2B6 (CYP2B6), and my major goal was to express, purify, and crystallize the enzyme. After some trial and error, I crystallized CYP2B6 (Y226H/K262R) in a complex with an imidazole inhibitor, which led to the first crystal structure of this enzyme (Gay, Shah, *et al.*, 2010). This structure was followed by crystallization and structure determination of many other complexes of CYP2B6 as well as other mammalian P450s in

complex with inhibitors, drugs, and monoterpenes, yielding an enormous amount of structural data (Shah *et al.*, 2011, 2012, 2015, 2016). As the saying goes, structures do not tell a complete story, and these data were always supplemented with functional and solution-biophysical approaches. Two specific projects during my eight years with Jim provided the most structural insights into CYP2B6. The first was the dual ligand complex with amlodipine that helped elucidate the substrate access channel leading to the active site and explain the enzyme's ability to bind and accommodate two drug molecules (Fig. 7A, Shah *et al.*, 2012). The second was realizing how many known substrates of CYP2B6 were halogenated and proposing the role of halogen-π bonds in the active site in substrate selectivity. The structural analysis indicated that halogen-π interactions might be unique to the CYP2B and 3A subfamilies (Fig. 7B) (Shah *et al.*, 2017).

The project that launched my independent career. Having studied cytochrome P450 enzymes for so many years, I was interested in seeking an independent faculty position to explore other members of the superfamily. I had already prepared a research proposal on structure-function relationships of important orphan P450s, including cytochrome P450 2U1 (CYP2U1), which are implicated in several diseases, along with a teaching statement necessary for faculty jobs. However, I had little knowledge that an unrelated project on P450s would change the course of my future research, at least for some time.

After our laboratory moved to the University of Connecticut, one of Jim's colleagues from Japan, Dr. Keiko Maekawa, now at Doshisha Women's College, Japan, approached us for assistance with analyzing structures of wild-type cytochrome P450 2C9 (CYP2C9) and genetic variants, *3 and *30, in complex with losartan that she had crystallized. The crystallographic data were collected and processed in Japan by a crystallography colleague who was not a P450 scientist. In

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addition to finding multiple binding sites for losartan in these structural complexes (Fig. 7C), I was amazed to see how the Ile-359 to Leu substitution in the *3 variant impacted the binding of additional losartan compared to the W.T., which may relate to reduced activity towards drug substrates in the clinic. During that time, I saw an advertisement at Albany College of Pharmacy and Health Sciences (ACPHS) for a faculty position specifically stating research interests in drug metabolism and pharmacogenomics. After the traditional interview process that included a chalk talk, I was hired as a tenure-track assistant professor, allowing me to launch an independent research program. In the first few months, I wrote a "communication" in *Biochemistry* on the structures of the CYP2C9 WT, *3, and *30 with Dr. Maekawa (Maekawa *et al.*, 2017). My laboratory soon crystallized the CYP2C9*2 variant, which proved crucial in obtaining an American Heart Association research grant in 2019 to study the impact of genetic polymorphisms of CYP2C9 on losartan and S-warfarin (Parikh *et al.*, 2020). Since then, I have determined the structures of several CYP2C9 variants with or without drugs. The supporting functional and biophysical studies provided valuable knowledge into the binding affinity, substrate turnover, and thermal denaturation of these variants (Parikh *et al.*, 2021). Overall, the research project served as a bridge between CYP2B and opportunities to explore other P450s.

Next steps: It was not long before I realized the importance of establishing new projects to sustain the laboratory in the long run. While in Jim's lab, I was introduced to some of his Pfizer colleagues who were interested in obtaining a crystal structure of cytochrome P450 2C8 (CYP2C8) in a complex with a drug glucuronide because of the role of such metabolites in drug-drug interactions. Compounds of interest were gemfibrozil and clopidogrel, and the main question was the nature of the glucuronide binding site. An important discussion with Dave Rodrigues from Pfizer in 2019 led to the project's inception. Additionally, I continued with my

interest in CYP2U1, which I had abandoned for several years to focus on the 2C subfamily. In my 5th year at ACPHS, we decided to take a fresh look and troubleshoot the expression and purification of CYP2U1 and were able to obtain diffracting crystals and determine the structure at 3.4 Å. We intend to take a multi-disciplinary approach to studying drug glucuronide complexes of CYP2C8 as well as structure-function relationships of 2U1. In conclusion, it has been a challenging and dynamic first few years of my independent academic career filled with new projects and ideas, not to mention the teaching and service commitment that comes with every faculty job.

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Conclusions

Reflections of James Halpert. In retrospect, my 45 years as an active biomedical researcher went fast. My heart still has a special fondness for my Ph.D. studies of presynaptic snake venom neurotoxins (Halpert and Eaker, 1975; Halpert *et al.*, 1976). However, the most important contributions I was fortunate to make to biochemistry and pharmacology reflect the hard work, creativity, resourcefulness, and resolve of younger colleagues focused on the P450 system. When I entered the field in 1978, it was obvious that amino acid residues within and outside the P450 active site must be crucial for enzyme function. However, no such residues had been identified; their role in species and individual differences in substrate metabolism was completely unknown, and solving a mammalian P450 X-ray crystal structure was a mere fantasy. Through the efforts of the individuals featured in this article and many others, our group achieved an atomic-level understanding of ligand binding and substrate turnover by CYP2B enzymes (Shah *et al.*, 2012; Halpert, 2020). We also revealed the importance of enzyme plasticity and its role in the thermodynamics of ligand binding (Shah *et al.*, 2015), as well as the role of halogen-π bonds in ligand recognition (Shah *et al.*, 2017), which were totally unanticipated before structures became available. Importantly, the plasticity of P450 enzymes inferred from the comparison of static X-ray CYP2B crystal structures has been demonstrated more directly with other P450s by NMR (Estrada *et al.*, 2013, 2014, 2016; Bart and Scott, 2017), and recent structures of CYP2C9 genetic variants have provided a mechanistic explanation of altered enzyme function (Maekawa *et al.*, 2017; Parikh *et al.*, 2020, 2021). Intriguingly, the biggest recent surprise regarding CYP2B enzymes came not from studies of protein structure but of genome structures from the laboratory of my collaborator Dr. Denise Dearing. Her group has discovered a novel CYP2B gene island in the woodrat *N. lepida*, which is comprised solely of variable numbers of duplicated genes most

similar to CYP2B37 (Greenhalgh *et al.*, 2022), and which may play an important role in allowing the animals to ingest diets high in toxic monoterpenes.

The individual co-authors worked on very different projects depending on interest and aptitude and the technologies available at the time. This article describes studies involving protein adducts, adrenal P450s, homology modeling, X-ray crystallography, directed evolution, NMR, and human P450 structure. Despite the different research foci, there were a number of commonalities in the training experience, including my own. Generally, after working initially on a more secure project, each person branched out into a new area where success was far from guaranteed. Often this occurred through collaborations, including traveling to outside laboratories for specialized training. Making presentations at international meetings and being introduced to world leaders, no matter how intimidating the experience, was expected. Each person who sought a subsequent career in academia was given the time and encouragement to develop an independent project that would be portable to a new institution. Now that I am retired, I look forward to following the contributions from Emily Scott, Santosh Kumar, Arthur Roberts, and Manish Shah as they explore and develop new facets of drug metabolism research. Their perspectives on future opportunities in selected areas are found below.

Perspectives

Human P450s in endogenous pathways (Emily E. Scott). The significance of P450 enzymes and their partner proteins in human disease has long been recognized. However, except for steroidogenic P450 enzymes, structural work on drug-metabolizing P450 enzymes has far outpaced that of P450 enzymes in endogenous pathways, which are frequently drug targets. Our structural work is now expanding to these enzymes in an effort to parallel the advances from Fred Guengerich's lab, which deorphanized several of them functionally. The combined

information should enable new pharmaceutical designs for a broad range of diseases. A recent advance in this area is the structural definition of the sterol 12-alpha-hydroxylase CYP8B1, which is involved in the production of bile acid cholic acid. Structure-based inhibitors of CYP8B1 show potential in treating type 2 diabetes and non-alcoholic fatty acid liver disease (Liu *et al.*, 2022). A second continuing area of interest is the interactions between cytochrome P450 enzymes and their redox partners, which have complex implications for the P450 function that we are still trying to understand (Loomis *et al.*, 2022).

Novel drug delivery modalities (Santosh Kumar). For over a decade, our research group has examined the role of CYP pathways in alcohol/ smoking-mediated HIV pathogenesis. Now, we are extending our research to develop novel drug delivery modalities to suppress HIV in the central nervous system (CNS) among alcohol drinkers and tobacco smokers. This work is important because HIV persists in the CNS and actively replicates, leading to HIV-associated neurocognitive disorders (HAND), which are further exacerbated in alcohol/tobacco users. To address this problem, we first developed lipid-based artificial nanoparticles to deliver an antiretroviral drug to the CNS (Gong *et al.*, 2020). However, artificial nanoparticles have limitations, such as the inability to target specific cells and lack of biodegradation, which raises safety concerns. Therefore, our group is developing natural nanoparticles called extracellular vesicles (EVs) as pharmacologically and clinically relevant drug delivery nanocarriers to treat HAND. Due to their natural ability to transport biomolecules (Rahman *et al.*, 2019), EVs have been recently recognized as novel biocarriers to deliver drugs to the CNS and combat neurological disorders, including HAND (Zhou *et al.*, 2022). In addition, EVs have intrinsic features of biocompatibility, stability, stealth capacity, the ability to overcome the blood-brain barrier, and inherent homing capabilities. These properties make EVs a promising tool for drug

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delivery to the CNS. In collaboration with other groups at my institution, our group is developing an EV-based drug delivery platform to deliver not only therapeutic drugs but also nutraceuticals as adjuvant therapy. Ultimately, we plan to extend the work to other neurological diseases such as Alzheimer's disease and stroke.

Transporter studies and beyond (Arthur G. Roberts). I am primarily interested in transporters and enzymes that hinder the development of safe and effective drugs. My laboratory wants to understand their mechanisms using novel approaches and innovative technologies. We focused on the P-gp drug transporter because of its unusual ability to tolerate amino acid substitutions, allowing me to use the NMR technology that I developed for CYP enzymes (Wilt *et al.*, 2017). The transporter is also a gatekeeper of the blood-brain barrier, a key driver of anti-cancer drug resistance, and a major cause of adverse drug reactions from cardiovascular therapeutics. In our initial investigations with P-gp, we developed a simple method for bioengineering liposomes with P-gp oriented in the lipid bilayer using thermodynamic principles that I learned as a graduate student (Ledwitch, Barnes, *et al.*, 2016). Orienting P-gp in the lipid bilayer has been vital for investigating its transport mechanism. For example, with AFM, we can monitor single transporter molecule dynamics involved in transport on a sub-nanometer scale (Sigdel *et al.*, 2018). Recently, using our P-gp bioengineering approach, we developed an oligonucleotide delivery vesicle that shows promise for targeting oligonucleotides to specific organs in mice (Jain and Roberts, 2021). We plan to use this technology to deliver plasmids that modulate the expression of crucial proteins and enzymes in drug disposition. Ultimately, we hope these investigations will lead to more efficient and less costly development of effective and safe therapeutics.

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Human P450s and clinical relevance (Manish Shah). I am interested in the structure-function relationships of multiple CYP enzymes that currently include 2C8 and 2C9 from the CYP2C subfamily and the so-called "orphan" CYP2U1. Using X-ray crystallography and other solution studies, our laboratory investigates the binding to these enzymes of relevant drug substrates or ligands to address clinical questions of significance. The aims are to (1) Elucidate the impact of less characterized genotypes of CYP2C9 on the therapeutic response to the highly prescribed anti-hypertensive medication losartan by combining basic and clinical research to aid in implementing pharmacogenomics guidelines and making personalized medication decisions. (2) Characterize how the binding and orientation of clopidogrel and gemfibrozil acyl-glucuronide metabolites in the active site of CYP2C8 inhibits the enzyme, as this could help develop safer medications devoid of drug-drug interactions in patients. (3) Determine the active site topology and structural adaptations of CYP2U1 upon binding endogenous substrates such as arachidonic acid and arachidonoyl serotonin, which may help in the design of new substrates or inhibitors of this relatively unexplored enzyme of physiological significance.

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Authorship Contributions

Wrote or contributed to the writing of the manuscript: Halpert, J.R., Stevens, J.C., Sklarz, G.D., Scott, E.E., Kumar, S., Roberts, A.G., Shah, M.B.

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Footnotes

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Figure Legends

Fig. 1. Metabolic scheme for bioactivation of chloramphenicol by CYP2B1 and release of the adduct *N*-e-chloramphenicol oxamyl lysine upon proteolytic digestion of the modified enzyme.

Fig. 2. Irreversible Type I spectral shift produced by the binding of 17α-ethynyl progesterone metabolite(s) to purified bovine adrenal cytochrome P450 21A2 (CYP21A2) (Stevens *et al.*, 1991). The figure was adapted with permission from Stevens JC, Jaw JY, Peng CT, and Halpert J, Mechanism-based inactivation of bovine adrenal cytochromes P450 C-21 and P450 17 alpha by 17 beta-substituted steroids. *Biochemistry* **30**:3649–3658. Copyright 1991 American Chemical Society.

Fig. 3. Docking of ligands into the active site of the CYP2B1 model. A: Progesterone docked into the active site of the CYP2B1 L209A mutant in a binding orientation leading to 21-OH progesterone. Key amino acid residues are shown in purple; Ala-209 is in green. Leu-209 (purple) is present in the WT enzyme, which leads to van der Waals overlaps with progesterone. B: BBT (*N*-benzyl-1-aminobenzotriazole) docked into the active site of CYP 2B1 in an orientation allowing for oxidation at the 1-amino nitrogen, leading to enzyme inactivation. When Gly-478 is replaced with Ala, van der Waals overlaps appear between the inhibitor and the Ala side chain. Key residues are in purple, and Ala-478 is in green. Such overlaps likely account for the resistance of G478A to inactivation by the chloramphenicol analog N-(2-*p*-nitrophenethyl)chlorofluoroacetamide (He *et al.*, 1992).

Fig. 4. Comparison of cytochrome P450 2B4 structures in the open (green) and closed, ligand-bound (blue) states, highlighting highly mobile elements of the B'C loop (orange) and F'-G helices (purple).

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Fig. 5. Schematic representation of the directed evolution approach to engineer mammalian CYP2B1 enzyme.

Fig. 6. My four research fields. During my research adventure, I investigated (A) Rieske (R) iron-sulfur domain movements with the cytochrome (cyt.) b_6f complex, (B) drug-drug interactions with cytochrome P450 3A4 (CYP3A4), (C) transport with P-glycoprotein (P-gp), and (D) targeted plasmid delivery with bioengineered liposomes. PDB IDs: CYP3A4: 1TQN; cyt. b_6f complex: 4PV1; P-gp: 5KPI; PC: 2BZC. Additional abbreviations: Ab, antibody; P.C., plastocyanin; PEG, polyethylene glycol; PS I, photosystem I. The figure was created with biorender.com.

Fig. 7. Structures of Human CYP2B6 and 2C9. (A) Dual ligand complex of CYP2B6 (Y226H/K262R) with amlodipine (violet sticks) bound that helped elucidate the substrate access channel from the active site near heme to the solvent region. (B) Structure of CYP2B6 (Y226H/K262R) bound with myrtenyl bromide (violet sticks) with bromine exhibiting halogen-π interaction with the aromatic phenylalanine side chains. (C) Structural overlay of CYP2C9 wild-type (green) and CYP2C9*3 (yellow) complexed with losartan. The *3 that represents isoleucine to leucine change at 359 affects neighboring tyrosine at 308 that impacts the residues in the active site including phenylalanine 476. The Phe576 side chain in *3 protrudes into the active site affecting the orientation of losartan compared to the wild-type complex. Losartan molecules are not shown for clarity purposes. Heme is shown in red stick representation.

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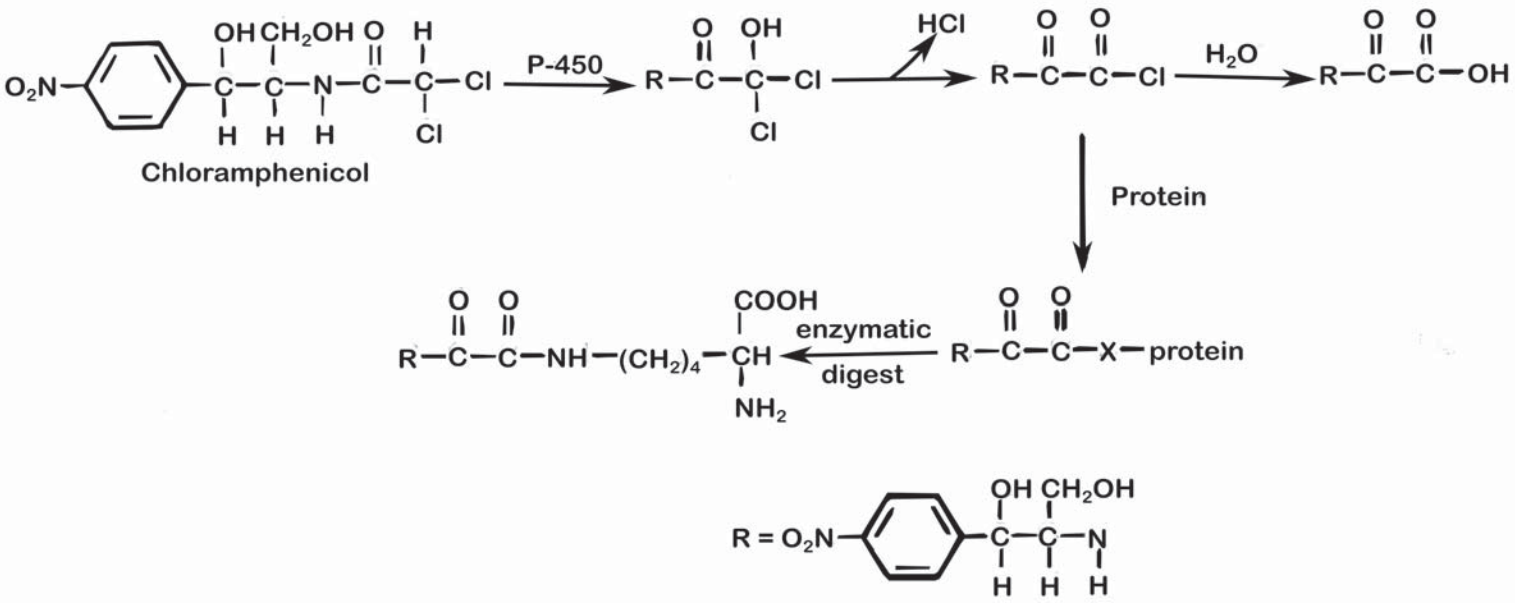

Figure 1

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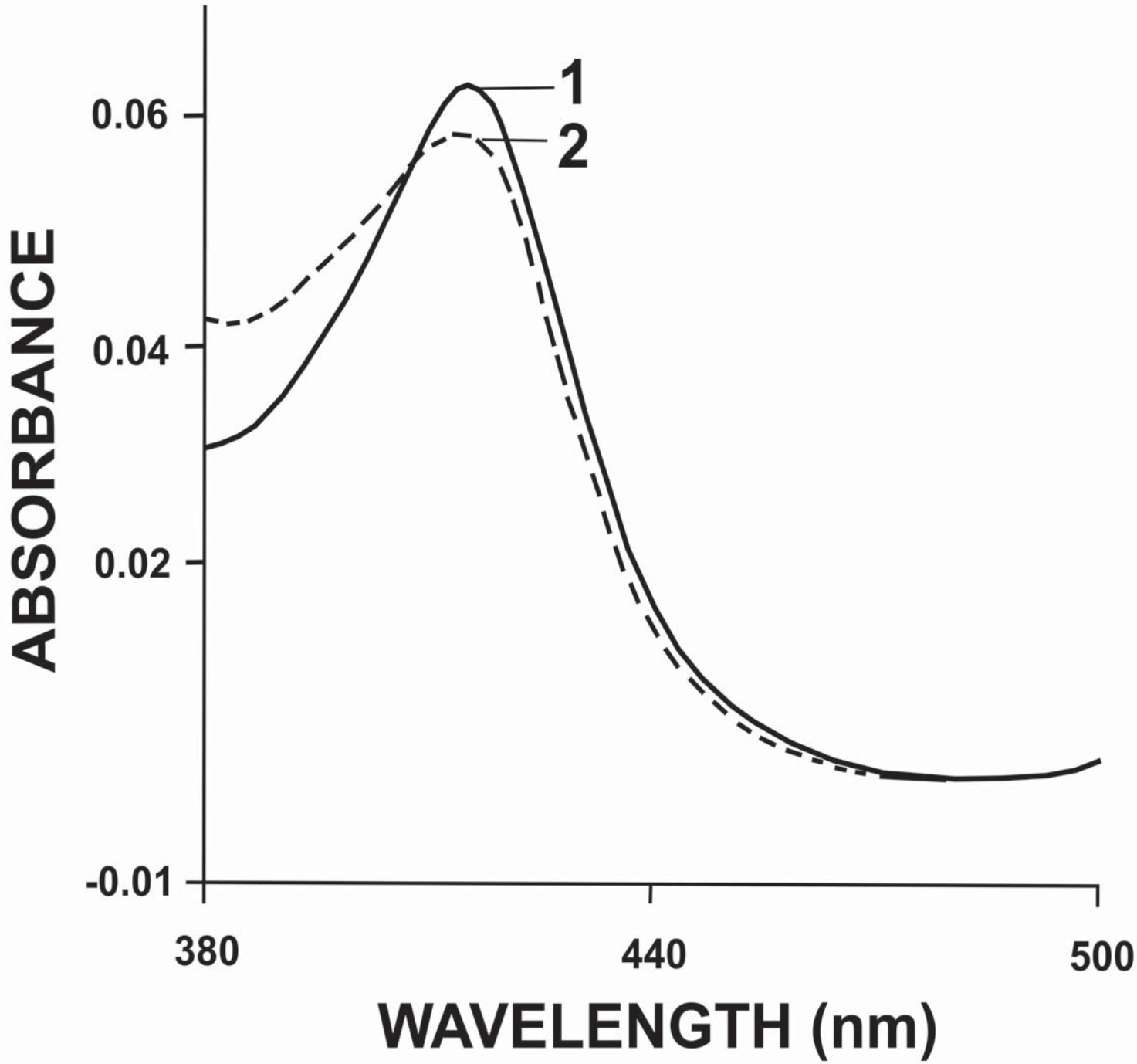

Figure 2

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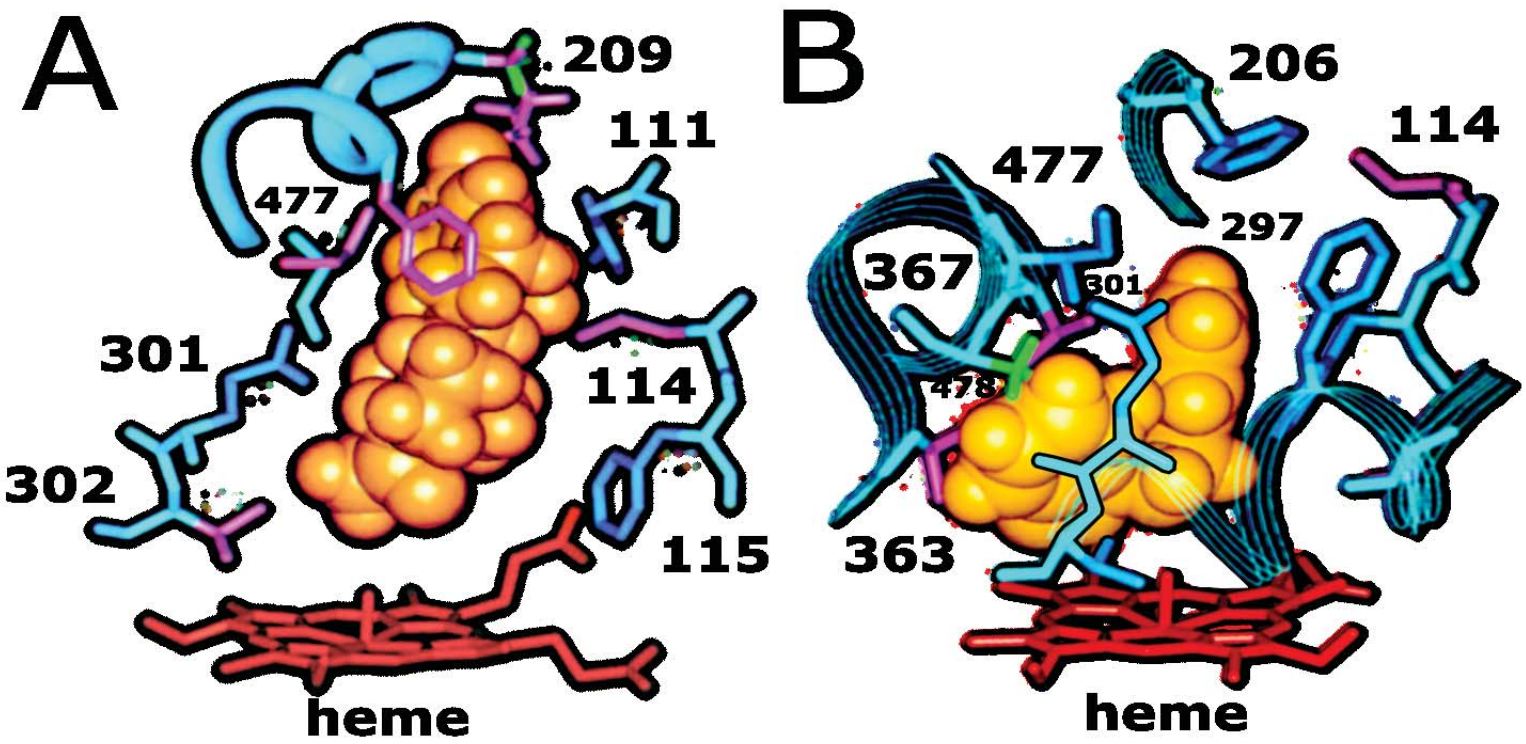

Figure 3

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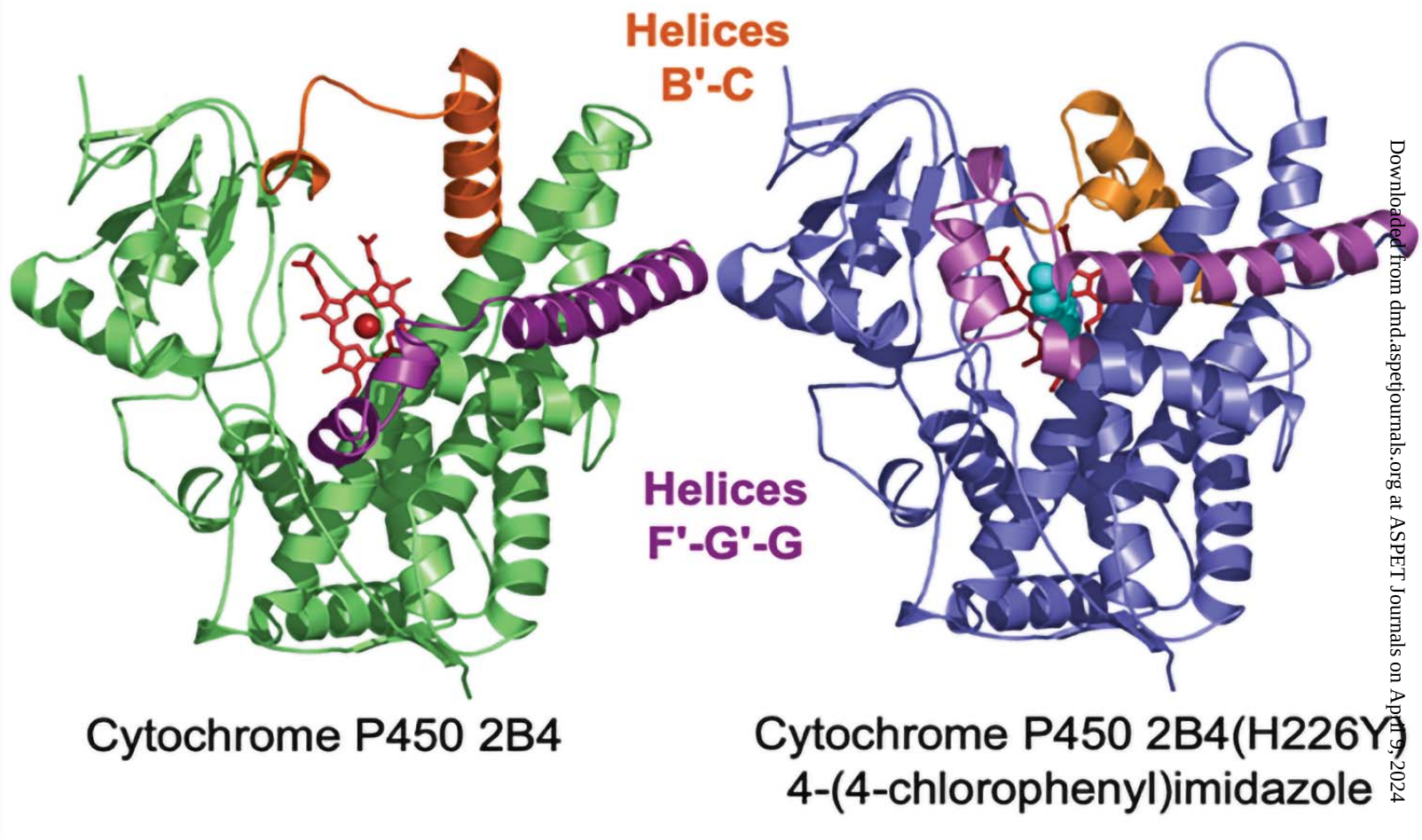

Figure 4

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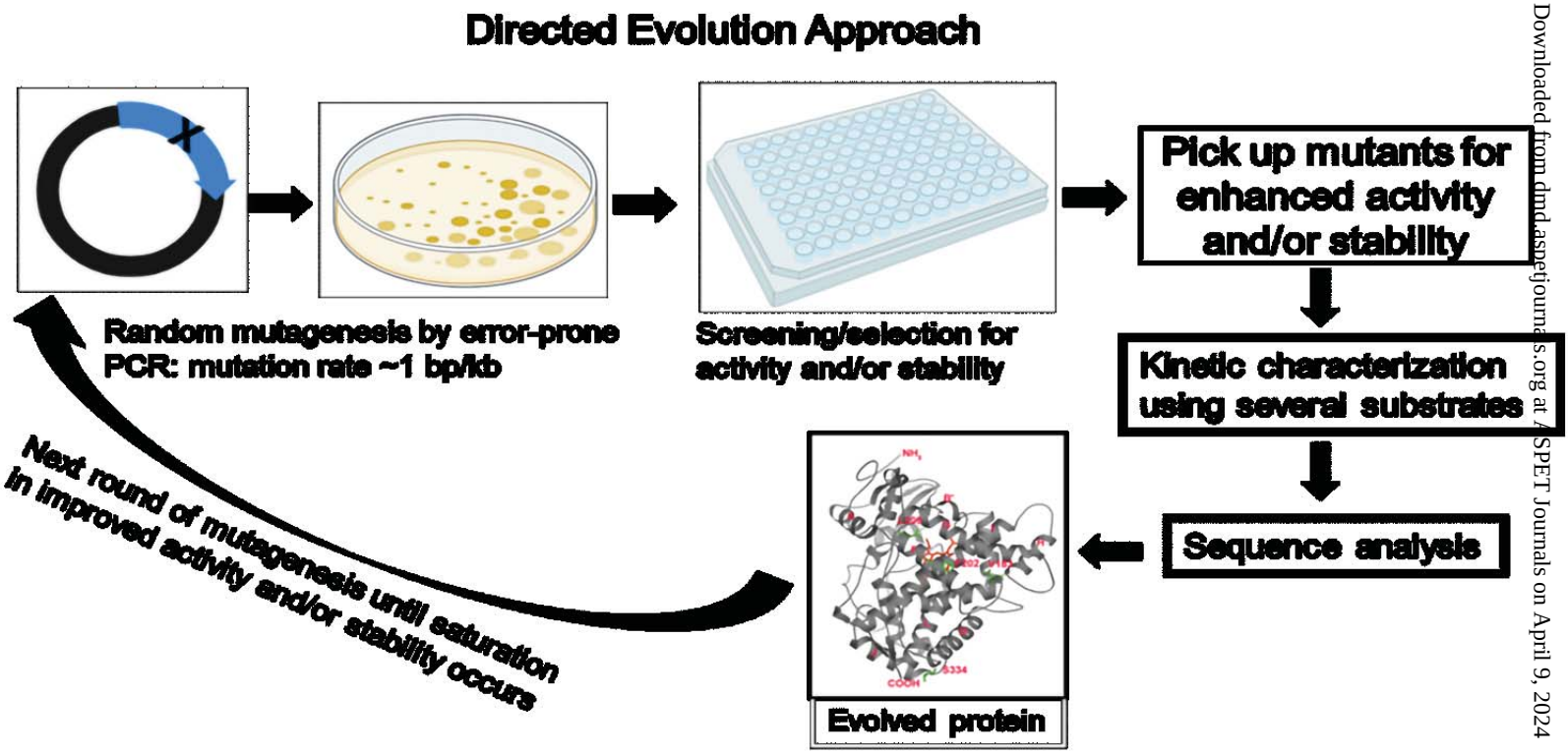

Figure 5

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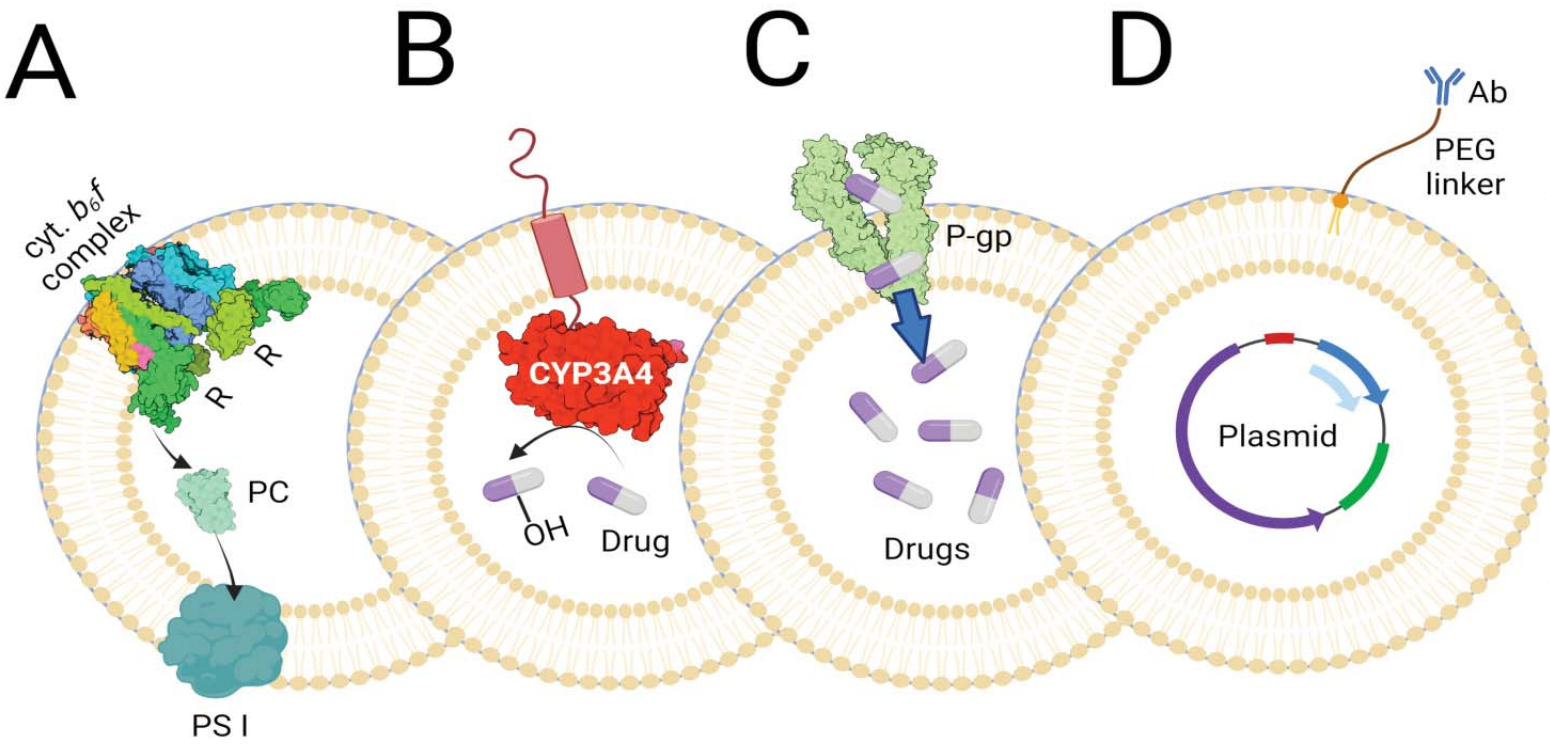

Figure 6

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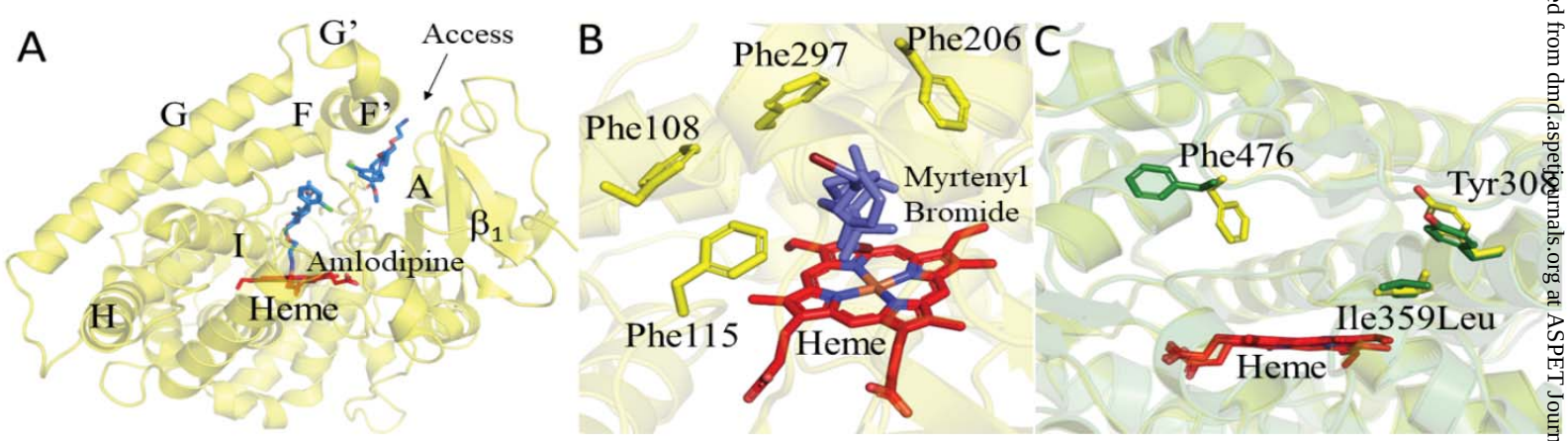

Figure 7