RESEARCH IN THE WANDLESS GROUP

The Wandless lab employs an interdisciplinary approach to studies of biological processes, combining synthetic chemistry with biochemistry, cell biology, and structural biology. However our core science is synthetic chemistry – the ability to create new molecules. Rather than focus on the development of new synthetic methods or the synthesis of molecules isolated from nature, my laboratory concentrates on molecules that possess novel functions. Specifically, we synthesize molecules that are designed to:

• provide insights into biological processes by perturbing specific proteins in cells
• utilize endogenous cellular proteins for entirely new purposes.
• probe the molecular interactions between small molecules and macromolecules.

Chemists bring a different perspective and techniques to biological problems, and while chemistry will certainly not provide answers to all biological questions, there are problems that stand to benefit from a very molecular approach. Some of the richest and most exciting research opportunities can be found at the interfaces of traditional disciplines, and our particular focus lies at the interface of chemistry and biology.

New Approaches for Conditional Control of Protein Function – Biological studies of mammalian development and physiology have been revolutionized by the ability to disrupt specific genes using homologous recombination in mice. However, interpretation of the phenotypes of transgenic or knock-out mice possessing null mutations is often clouded by early embryonic lethality as well as cellular and molecular compensation for the absence of a gene during development. Methods for conditional gene inactivation have been developed, but these methods are typically slow and irreversible. There is a widespread need for a general method to reversibly target the protein product of a specific gene rather than the gene itself.

In collaboration with Jerry Crabtree's lab here at Stanford, we recently developed an experimental system in which the stability of a specific protein depends on the presence or absence of a synthetic, cell-permeable organic molecule. Rapamycin is a natural product that binds simultaneously to the FK506-binding protein (FKBP) and to the mTOR/FRAP protein. We modified rapamycin to create a derivative called MaRap that binds tightly to FKBP, however MaRap cannot bind to the biologically relevant effector protein, mTOR/FRAP. A small, 89-amino acid fragment of mTOR/FRAP called FRB comprises the rapamycin binding domain, and MaRap cannot bind to FRB. However, the introduction of three specific mutations in FRB provides a protein called FRB* that can accommodate the larger methylallyl group of MaRap. Fusion of the FRB* sequence to a gene of interest produces a chimeric protein containing the FRB* domain, and our studies demonstrate that proteins fused to FRB* are constitutively degraded by the proteasome. Addition of MaRap recruits endogenous FKBP to the FRB* fusion protein, and this resulting trimeric complex is protected from degradation, effectively stabilizing the protein of interest. This new technique allows rapid and reversible elimination of a specific protein either in cell culture or in mice. See publications 26, 30, 32, and 33 on the "publications" webpage for more information.

Protein-Protein Interactions & Bifunctional Molecules – One of the primary goals of my laboratory has been the design, synthesis and evaluation of synthetic molecules that are capable of binding to two different proteins. These molecules are typically comprised of two ligands for their respective proteins, and the ligands are linked by a covalent tether. In cases where the tether between the ligands is relatively long, both proteins can simultaneously bind to the bifunctional molecule to form a trimeric complex. The formation of a trimeric complex
creates an environment wherein interactions between the two proteins are possible. These nascent protein-protein interactions may contribute either favorably or unfavorably to the overall stability of the ternary complex, and we have shown that these protein-protein interactions may endow the bifunctional molecules with biophysical and biological properties that are significantly different from the monomeric ligands that comprise them. In one of our first studies, we demonstrated that a trimeric complex is more stable than one might expect based on the stabilities of the two constituent dimeric complexes. Please refer to reference 16 in the publications list for more information.

Bifunctional molecules that possess short tethers create a very different environment. In this case, unfavorable protein-protein interactions significantly destabilize the ternary complex relative to the two dimeric complexes, and the bifunctional molecule will partition between its two protein receptors as a function of the respective dissociation constants as well as the concentrations of all three species. We have capitalized on this behavior to design a bifunctional molecule containing one ligand that is capable of inhibiting an essential cellular enzyme and displaying cytotoxicity. However, the other ligand binds tightly to a very abundant cellular protein called a presenter protein, and this binding event does not elicit any measurable cellular response. Thus, in the presence of both proteins, the bifunctional molecule is effectively "detoxified" by the presenter protein.

However, if the presenter protein is either absent or binds weakly to its ligand, the bifunctional molecule is free to inhibit the essential enzyme and cause cytotoxicity. Bifunctional molecules such as these display context-dependent biological activity. Their ability to cause a biological response depends on the presence or absence of a biologically silent presenter protein rather than the target protein. As a result, we have decoupled a molecule's ability to elicit biological activity from the presence or absence of the target protein. Two different cells that possess identical amounts of the same target protein (but different presenter proteins) will respond differently to a bifunctional molecule. With a knowledge of a cellular environment, which is increasingly available using genomic and proteomic techniques, we can design and synthesize bifunctional molecules that display predictable selectivity for different proteins or cell types. Using a bifunctional molecule capable of inhibiting dihydrofolate reductase, we have demonstrated selective cytotoxicity toward malaria parasites but not against mammalian cells that possess the protective protein as well as the target protein. We have demonstrated this detoxification strategy in cell-free assays using purified proteins, cell-based assays against the *Plasmodium falciparum* malaria parasite, and using *Plasmodium*-infected mice. For more information, please refer to publication 25 in the publications list.

From the outset of these studies, we have been working to understand the molecular details that give rise to the unique properties of these bifunctional molecules. One line of investigation has focused on the nature of the putative protein-protein interactions. We have used a variety of analytical techniques to probe these interactions including NMR spectroscopy, mass spectrometry, fluorescence anisotropy, and surface plasmon resonance. Another useful aspect of these bifunctional molecules is the predictive power that is provided when information about the constituent protein-ligand interactions is available. We have formulated quantitative models that incorporate multiple equilibria to account for all possible molecular species (individual molecules as well as all dimeric and ternary complexes) and potential dissociation constants. These models can be used qualitatively to discern whether or not a trimeric complex contributes to a particular binding equilibrium. Furthermore, fitting of experimental data to the model provides quantitative information about the stability of participating trimeric complexes. Our ability to model equilibria involving the trimeric complexes has been very valuable both for
designing experimental systems as well as for providing quantitative data following experiments. See publications 29 and 22 for more on the analysis of protein-protein interactions, and see publication 27 for a detailed description of the quantitative model.

We are pursuing several additional uses for bifunctional molecules as probes of cellular signaling pathways. With Takanari Inoue in Tobias Meyer's lab, we are using new rapamycin derivatives to rapidly localize signaling proteins to the inner leaflet of the plasma membrane in order to study the roles of these various domains in different signaling networks. Many cellular signaling processes are initiated at this location, and we use this rapid perturbation method to probe the functional roles of different proteins (small G-proteins and kinases) in specific signaling pathways. The use of these cell-permeable small molecules to perturb cellular signaling events enjoys several advantages over traditional genetic approaches, the most significant of which is the ability to measure real-time cellular responses in living cells with very fast temporal resolution. See publication 31 for more information.

**Synthetic Probes of Mammalian Checkpoints** – Another significant focus of our research has been the elucidation of cell cycle checkpoints that monitor microtubule integrity. Dozens of different small molecules, both natural products and synthetic compounds, bind to microtubules and interfere with their normal functions. Examples include taxol, vincristine, and epothilone, and many of these tubulin poisons are used as anticancer drugs. Early pharmacologic models postulated that these drugs caused significant damage to the cellular cytoskeleton, thus causing cytotoxicity in rapidly dividing cells such as cancer cells. Current pharmacologic models postulate that these drugs inhibit a microtubule-specific process called dynamic instability.

However, this model only begs the question of how these drugs inhibit microtubule dynamics, and more significantly, how cells monitor microtubule dynamics and sense that microtubule function is impaired. We set out to test this model using synthetic chemistry to prepare phomopsin B and ustiloxin D. Phomopsin is one of the most potent anti-tubulin molecules yet discovered, and through the synthesis of the natural product as well as a variety of closely related nonnatural products, we are testing these models of antimitotic drug action. We are correlating in vitro measurements of microtubule dynamic instability with the cellular effects of these molecules. A high degree of correlation provides evidence in favor of the dynamic instability model, and a lack of agreement point to an alternative model. We plan to use phomopsin and its variants to gain insights into the molecular mechanisms underlying this checkpoint. Publications 28, 24, 18, 17, and 15 address the synthetic challenges presented by these natural products.