Spotlight

Nothin' but a GPCR Thing

From a structural biologist's perspective, receptors remain the most elusive family of cellular proteins. Still, eager laboratories take on this challenge because many of these proteins represent a first flag in signaling cascades or are current or potential drug targets. Getting a look at the atomic topology of these molecules is often thwarted by the hydrophobic nature of transmembrane domains, but this is only if a researcher is lucky enough to obtain the milligram quantities of pure protein that X-ray crystallography requires. Now, Sarkar *et al.* (*Proc. Natl. Acad. Sci. U.S.A.* 2008, *105*, 14,808–14,813) take a new angle on these misbehaving molecules by randomly tinkering with the primary sequence of a G-protein-coupled receptor (GPCR) in search of a mutant with increased expression and stability. Their iterative method is relatively simple, yet it has broad implications for the large number of proteins that fly below the structural biology threshold.

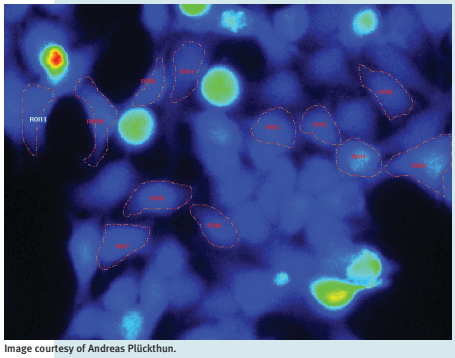


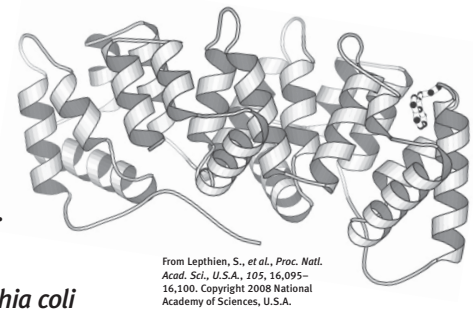
Image courtesy of Andreas Plückthun.

To set up the system, the authors fused their receptor of interest, the neurotensin receptor-1 (NTR1), to tags that target the protein to the *Escherichia coli* inner membrane. Then, using a specialized permeabilization protocol that allows small molecules to reach the inner membrane of a bacterium without killing it, they added a fluorescent ligand of the receptor, and any expressed and properly folded NTR1 protein was thereby bound. Using a fluorescence-activated cell sorter (FACS), they recovered those bacteria that possessed functional protein and grew them in media. With this procedure optimized, the NTR1 receptor was mutagenized and the FACS was programmed to recover increasingly fluorescent bacteria, indicative of more protein being expressed in the mutant strains. After several rounds of recovery, mutagenesis, and growth, one clone displayed ~10 fold higher signal than the starting NTR1 strain. This mutant protein displayed higher accumulation in not only *E. coli*, but also a yeast expression system and a human kidney cell line. Studies in human cells verified that the mutant GPCR could still perform proper signaling, whereas biochemical purifications yielded more protein and a receptor with higher thermal stability when solubilized in detergent. The researchers then took an interesting direction by using the screening method that they developed to look for receptors with altered ligand-binding properties. By using the same fluorescent agonist during screening, but competing with excess antagonist molecule, they isolated a single amino acid mutant that resists the antagonist but fully binds agonist. Because many drugs function by acting as an agonist or antagonist to a cellular receptor, this methodology should be widely applicable to many protein targets. This study shows that high-throughput mutagenesis and screening, more commonly a tool of model organism biologists, can come to the rescue for structural biologists and pharmacologists alike. **Jason G. Underwood, Ph.D.**

Making Tryptophan Blue

Endowing proteins with a fluorescent tag for detection purposes has become a cornerstone of investigations into protein biology. Of the many methods that have been developed, including the use of various protein engineering approaches and post-translational modifications, the procedures are often laborious and multi-step and can result in structural perturbations that can affect protein function. Lepthien *et al.* (*Proc. Natl. Acad. Sci. U.S.A.* 2008, *105*, 16,095–16,100) now report the use of azaindoles ((Aza)Ind) to convert proteins into their fluorescent blue counterparts with minimal effects to protein structure.

Indoles are present in tryptophan residues, which are attractive targets for modification because small structural changes can translate to large changes in spectral properties. The genetic code for amino acid tryptophan can be expanded by compounds (4-Aza)Ind and (5-Aza)Ind, in which an endocyclic –CH– of the indole ring is replaced with a nitrogen. These compounds exhibit a strong red-shifted fluorescence ("blue fluorescence") in aqueous solution when compared with that of indole itself. When (4-Aza)Ind or (5-Aza)Ind was supplied into synthetic growth media, bacterial cells were able to uptake the compound at a high rate and metabolically transform it into an (Aza)Trp, which was subsequently incorporated into cellular proteins. After the fermentation experiment, the auxotrophic *Escherichia coli* cells (*i.e.*, cells unable to synthesize tryptophan itself) were able to induce a blue glow when illuminated with UV light. However, it still remained to be determined to what extent the bacteria could incorporate these compounds as tryptophan analogs in their proteomes. On the other hand, the incorporation in single recombinant proteins is simple and straightforward. For example, the recombinant human protein anxA5, whose fluorescence emission spectra is dominated by its single tryptophan residue, was expressed in the bacteria and purified, and examination by gel electrophoresis showed highly fluorescent bands upon exposure to UV light. Mass spectrometry analyses unambiguously confirmed the high level of incorporation, and fluorescence emission profiles revealed dramatically red-shifted emission maxima for the (Aza)Trp-containing proteins. Expectedly, small differences between the proteins containing (Aza)Trp and Trp are found in related protein structures as measured by circular dichroism. Eva J. Gordon, Ph.D.



From Lepthien, S., *et al.*, *Proc. Natl. Acad. Sci., U.S.A.*, *105*, 16,095–16,100. Copyright 2008 National Academy of Sciences, U.S.A.

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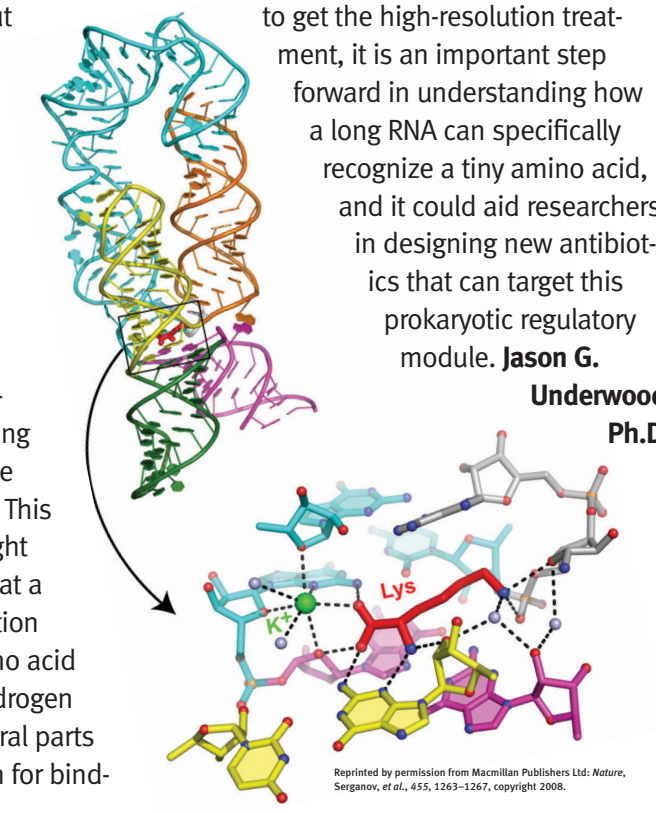
Locking onto a Lysine

Riboswitches get their futuristic name from their impressive characteristics. These structured messenger RNA (mRNA) segments function as gene regulatory switches that turn on or off by the binding of an effector ligand. When the small molecule is bound, the RNA structure can favor new secondary structure features within the switch or a neighboring RNA stretch. This can mean a change in the mRNA synthesis or translational efficiency of a nearby protein-coding gene. In bacteria, many of the discovered switches are elegantly situated proximal to the very genes that produce or transport the metabolite that is sensed by the RNA. This is analogous to the classic Lac operon taught in every introductory biochemistry course, but without a protein as a secondary messenger. One puzzling riboswitch ligand is lysine, a positively charged amino acid that is often found at binding interfaces between RNA and protein. How can lysine be recognized in a highly specific manner to then alter the expression of a dehydrogenase enzyme involved in synthesis of a lysine precursor? To get at this question, two groups solved the 3D structures of a bacterial lysine riboswitch with and without the bound ligand (Garst *et al.*, *J. Biol. Chem.* 2008, *283*, 22347–22351 and Serganov *et al.*, *Nature* 2008, *455*, 1263–1267. Although both groups found a similar architecture, the latter group's higher resolution structure provides details that make this riboswitch a particularly interesting ligand-binding RNA. The Serganov *et al.* high-resolution structures uncovered addition details of how lysine is recognized and point toward how antibacterial drugs that mimic lysine might target this important regulatory RNA.

The overall architecture of the 174 nucleotide lysine-binding RNA displays acrobatic secondary and tertiary interactions to stabilize the structure, but few structural changes occur during ligand binding, indicative of a largely preorganized riboswitch structure. The lysine binds to lock in the structure with a binding site nested in the core of the RNA. This switch uses a tight cleft positioned at a five-helical junction to bind the amino acid with specific hydrogen bonding to several parts of the RNA chain for binding specificity. A critical potassium ion coordinates with numerous junction nucleotides to stabilize the structure but also provides recognition for the carboxyl group on lysine. Interestingly, biochemical experiments showed that replacement of potassium with sodium in the lysine riboswitch resulted in decrease of lysine binding affinity. The close-up look at the lysine binding pocket also helped the authors infer how lysine mimics might target the riboswitch and how specific mutations in the RNA lead to loss of riboswitch function. While this is not the first riboswitch to get the high-resolution treatment, it is an important step forward in understanding how a long RNA can specifically recognize a tiny amino acid, and it could aid researchers in designing new antibiotics that can target this prokaryotic regulatory module. **Jason G. Underwood, Ph.D.**



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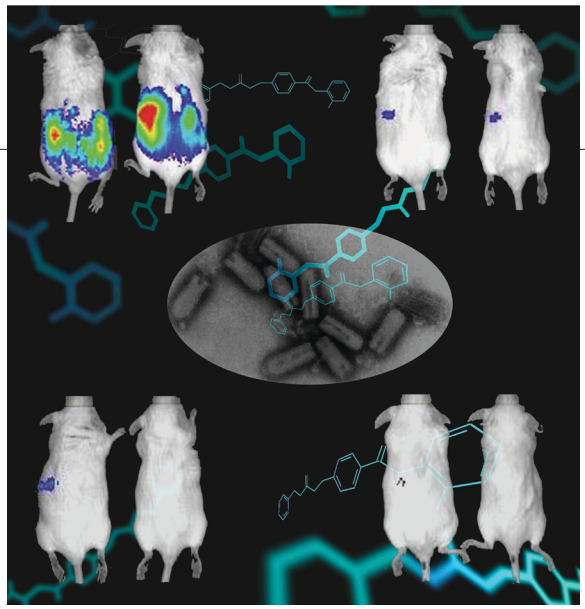
Spotlight

Inhibitors and Viruses Join Forces



Nguyên, T. L.-A., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, *105*, 14,981–14,986. Copyright 2008 National Academy of Sciences, U.S.A.

Oncolytic virotherapy, in which the normally unwanted, destructive behavior of viruses is hijacked and redirected to tumors, is an exciting new strategy in the war against cancer. However, the innate antiviral response unleashed by cells when a virus is detected, although often compromised in tumor cells, has nevertheless emerged as an obstacle to the success of this promising approach. Now, Nguyên *et al.* (*Proc. Natl. Acad. Sci. U.S.A.*, 2008, *105*, 14,981–14,986) present an innovative strategy that employs histone deacetylase inhibitors (HDIs) to help oncolytic viruses dodge the antiviral response.

As molecules that can influence epigenetic chromatin modifications, histone deacetylase inhibitors interfere with the antiviral response by preventing the transcriptional activation of antiviral genes, called interferons, in response to infection. The authors hypothesized that pretreatment of tumors with HDIs might cripple the ability of the tumor to mount an antiviral response, thus empowering the virus to deliver its destructive forces specifically at the tumor site. Indeed, when cancer cell lines, primary human tumor tissue, and mouse cancer models were treated with HDIs and infected with oncolytic virus, virus replication and spread dramatically increased, and virus-induced apoptosis in malignant cells was enhanced. Investigation into the mechanism of HDI activity indeed revealed that the compounds inhibit expression of interferon and of interferon-inducible genes. Notably, the combination of HDI treatment and oncolytic viral infection appears to have a synergistic effect on cell death. An especially appealing aspect of this strategy is the fact that HDI activity is reversible, suggesting that by simply altering the amount of HDI administered, the magnitude of oncolytic viral therapy could be tuned according to patient needs. In addition, the fact that both oncolytic viruses and HDI inhibitors have already separately begun clinical trials indicates that clinical testing of this promising approach can proceed in an accelerated manner.
Eva J. Gordon, Ph.D.

Profiling Penicillin

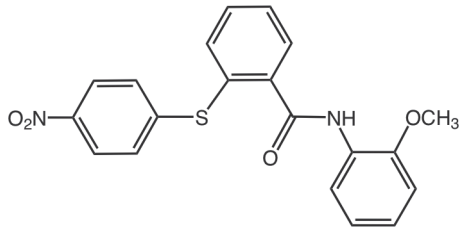
β-Lactams are a pivotal class of antibiotics that function by inhibiting penicillin binding proteins (PBPs), key enzymes in the biosynthesis of the bacterial cell wall. The widespread use and limited number of β-lactams have led to the emergence of bacterial strains resistant to this drug class, posing an alarming threat to our ability to combat such pathogens. Despite the vast number of studies on PBPs, surprisingly little is known about the regulation and function of many of these proteins. Using a technique called activity-based protein profiling (ABPP), Staub and Sieber (*J. Am. Chem. Soc.* 2008, *130*, 13,400–13,409) gain a new perspective on the proteins targeted by β-lactams.

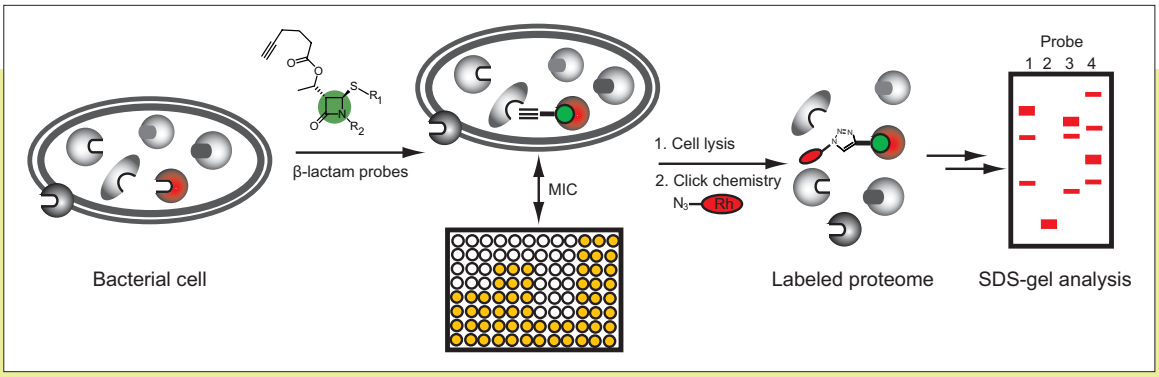
ABBP overcomes many limitations of previous methods used to explore PBPs, which suffered from tedious procedures, hazardous materials, and inefficient β-lactam probes. When a short alkyne handle is appended to the β-lactam core, a β-lactam probe is created that can be added directly to cell lysates or live bacteria, where it can bind to PBPs. After enzyme binding, the β-lactam probe can be reacted selectively at the alkyne moiety to install a fluorescent tag. The tag then enables detection by gel electrophoresis and fluorescence scanning and identification by mass spectrometry of any PBPs that

Targeting an HIV Axis of Evil

In the nearly 30 years since the emergence of AIDS, >20 million people have died from the disease. Despite tremendous progress in our understanding and treatment of AIDS, the toxicity associated with current drug treatments, which are dominated by inhibitors of HIV-1 reverse transcriptase or protease, along with the development of resistant viral strains begs the development of improved therapeutic options. To this end, Nathans *et al.* (*Nat. Biotechnol.* 2008, *26*, 1187–1192) report the identification of a small molecule that targets Vif, an HIV-1 regulatory protein that is essential for viral replication.

Vif is known to target the human DNA-editing enzyme APOBEC3G (A3G), down-regulating A3G levels and thwarting its ability to inhibit viral replication. To find small molecules that disrupt the Vif-A3G interaction, the authors screened 30,000 compounds for their ability to increase A3G levels in HIV-infected cells. From >500 primary hits, a series of secondary screens led to the identification of the Vif antagonist RN-18. Investigation of the mechanism of RN-18 activity revealed that the compound specifically down-regulates Vif levels and increases A3G levels in virus-producing cells and in virus particles, which effectively prevents the virus from replicating. It was further shown that RN-18 is not a general proteasome inhibitor, an important distinction given that Vif promotes the degradation of A3G by the proteasome. Moreover, consistent with the DNA editing function of A3G, the presence of RN-18 results in enhanced cytidine deamination of the viral genome, resulting in the production of a less virulent virus. The discovery of a compound that effectively targets the Vif-A3G axis offers a new therapeutic strategy for AIDS treatment, as well as valuable new pharmacological tools to probe Vif function and HIV biology. **Eva J. Gordon, Ph.D.**





interacted with the probe. With this approach, several known β-lactams as well as novel synthetic β-lactam structures were exposed to various bacterial strains, and targeted proteins were identified. In addition to known PBP targets, several additional enzymes were identified, including some proteins important for resistance and virulence. This suggests that the β-lactam core, when decorated appropriately, could be developed as an inhibitor of protein classes distinct from those involved in cell wall synthesis. This is especially relevant considering that inhibiting these types of enzymes may be a promising strategy for combating antibiotic-resistant strains. **Eva J. Gordon, Ph.D.**

A Beetle, a Bacterium, and a Fungus

Symbiotic relationships between microbes and plants and animals offer a mutually beneficial environment for both the host and the microorganism with which it coexists. Southern pine beetles participate in a beneficial symbiosis with the fungus *Entomocorticium* sp. A, which provides a source of nourishment for the beetle's larvae. However, this relationship can be threatened by another fungus, *Ophiostoma minus*, which can out-compete *Entomocorticium* sp. A and perturb larvae development. Scott *et al.* (*Science* 2008, *322*, 63) now report that the beetle cleverly engages in a second symbiotic relationship, this time with **a bacterium**, to protect its fungal partner.

Beetles carry *Entomocorticium* sp. A in a specialized compartment on the body called a mycangium, and they excavate galleries in pine trees into which they inoculate the fungus. Unexpectedly, scanning electron microscopy revealed a significant presence of the Gram-positive bacteria actinomycetes in both the mycangium and the galleries. This coexistence of actinomycetes was confirmed upon examination of culture isolations from individual beetles, many of which yielded actinomycetes exhibiting a red morphotype. In symbiont pairing assays, this red morphotype appeared to produce a substance that inhibited the growth of *O. minus*, with only minor effects on *Entomocorticium* sp. A. Identification and characterization of the compound responsible, using chemical and spectroscopic methods, revealed it to be a previously unknown polyene peroxide, which the authors named mycangimycin.

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Antifungal assays using purified mycangimycin confirmed its inhibitory capability, demonstrating that *O. minus* was 20× more sensitive to the compound than *Entomocorticium* sp. A. Because fungus farming ants also use actinomycetes to protect their fungal symbionts from pathogens, this type of trio may be a common strategy used by hosts to maintain their mutually beneficial relationships. Notably, these relationships could be a valuable source for the discovery of new antibiotic agents. **Eva J. Gordon, Ph.D.**

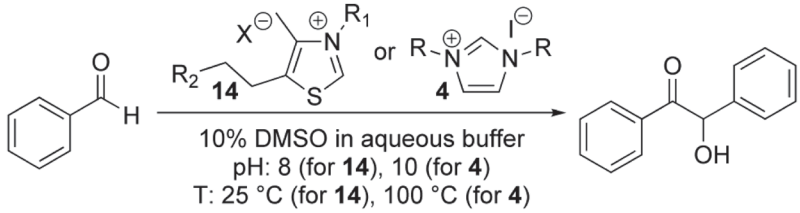
Menaquinone—In More Ways Than One

Menaquinone, also known as vitamin K_2, is a lipophilic compound that is a key component of the electron transfer pathway and an essential metabolite in many prokaryotes and eukaryotes. Whereas humans get most of their menaquinone through their diet, most bacteria must make this important compound themselves. Puzzlingly, the genes involved in menaquinone biosynthesis in *Escherichia coli* do not appear to exist in other bacteria known to produce menaquinones. Now, Hiratsuka *et al.* (*Science* 2008, *321*, 1670–1673) report the delineation of an alternative menaquinone biosynthetic pathway used by several microorganisms, including such pathogenic bacteria as *Helicobacter pylori* and *Campylobacter jejuni*.

Beginning with a bioinformatics approach, the authors screened bacterial genome databases for organisms that lacked orthologs of the genes involved in the known menaquinone biosynthetic pathway. Then, to find candidate genes, orthologous genes were estimated as reciprocal best-hit pairs using the BLAST (Basic Local Alignment Search Tool) program. By comparing candidate genes with those in microorganisms in which the known menaquinone pathway operates, they identified ~50 genes, and from these, exclusions based on putative functions of the candidate gene products slimmed the candidates down to four. Next, a series of gene disruption experiments revealed that mutants disrupted at each of the four candidate sites did indeed require menaquinone 4 for growth. Additional mutagenesis experiments designed to identify other genes involved in the alternative pathway revealed that chorismate is the branching point between the alternative pathway and the known pathway. Finally, analysis of the growth characteristics of the mutants along with the identification of various intermediates produced by them facilitated the generation of a detailed outline of the pathway from chorismate. Because this alternative pathway does not appear to exist in eukaryotes, it is an exciting potential target for drug discovery efforts against certain pathogenic organisms. **Eva J. Gordon, Ph.D.**

Artificial Coenzymes for Artificial Enzymes

Enzymes, often in partnership with small-molecule accomplices called coenzymes, are remarkable in their ability to achieve significant rate enhancements for a wide range of chemical reactions. Also remarkable has been the progress chemists have made in creating artificial enzymes that too can accelerate reaction rates. Thiamine diphosphate is a coenzyme that facilitates many important biological reactions by functioning as a stable equivalent of an acyl anion. Zhao *et al.* (*J. Am. Chem. Soc.* 2008, *130*, 12,590–12,591) now demonstrate the use of thiazolium and imidazolium ions as coenzyme mimics, along with modified polyethyleneimines (PEIs) as artificial enzymes, to catalyze the benzoin condensation.



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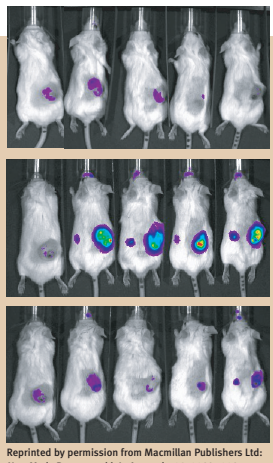
In the benzoin condensation, two aromatic aldehydes, in this case both benzaldehyde, react with the help of a nucleophile to yield the hydroxyketone benzoin. Three different modified PEIs were synthesized as enzyme surrogates, designed such that in aqueous solution they are capable of accommodating the coenzyme mimics and the benzaldehydes in a nonpolar interior, similar to the hydrophobic core found in many natural enzymes. Indeed, when benzaldehyde was combined with the PEIs and the coenzyme mimics, benzoin was generated with up to 10^3-fold acceleration in reaction rate. The most effective catalysts were those containing hydrophobic side chains on both the PEI and the thiazolium or imidazolium coenzyme, presumably because of their enhanced ability to engulf the substrate in a water-excluded environment. In addition, polycationic PEIs were more effective catalysts than neutral ones, possibly because of the electrostatic stabilization of the negative charges present during the reaction. Notably, this difference was more striking with the less efficient imidazolium cofactors, which needed to be tested at higher pH, than with the thiazolium cofactors, underscoring the importance of the electrostatic stabilization capabilities of the PEI. Development of such artificial enzymes contributes greatly to our understanding of the factors important in both chemical and biological catalysis. **Eva J. Gordon, Ph.D.**

Shielded Regulation

In addition to their many talents as protein activators and inhibitors, small molecules have found various other applications in controlling gene expression and protein function. In one system recently demonstrated in cultured cells, a small, inherently unstable protein domain is partnered with a small molecule, termed Shield-1, that is capable of stabilizing it. In the absence of Shield-1, fusions of the destabilizing domain with other proteins leads to their degradation by the proteasome, but in the presence of the small molecule, the fusion protein is stable and functional. Now, Banaszynski *et al.* (*Nat. Med.* 2008, *14*, 1123–1127) demonstrate the power of this system in living mice.

As an initial test, the destabilizing domain was fused to luciferase and stably integrated into a colon cancer cell line, which was then xenografted into immunodeficient mice. Indeed, luciferase activity was detected in the mice by *in vivo* bioluminescence imaging, and the activity was dependent on the presence and concentration of Shield-1. Next, the system was tested with interleukin-2 (IL-2), a physiologically important cytokine that has demonstrated anti-tumor activity. Following a similar process as with luciferase, colon cancer cells were stably transfected with destabilizing domain-IL-2 fusion proteins, but in this case the cells were xenografted into mice capable of producing immune cells, and the ability of the cells to establish tumors was monitored. Remarkably, while tumors grew unchecked in control mice, mice treated with Shield-1 showed significantly reduced tumor burden. Finally, a third system was tested, this time using a viral gene delivery system to supply tumor necrosis factor-α (TNF-α), a cytokine with anticancer activity, directly to a tumor site. Mice injected with the virus expressing a destabilizing domain-TNF-α fusion protein, followed by treatment with Shield-1, saw significantly enhanced survival rates compared with controls. This system demonstrates the power of using genetic methods combined with small-molecule regulators to explore biological processes and enable novel therapeutic applications. **Eva J. Gordon, Ph.D.**



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