Chemical Genetics: Exploring the Role of the Proteasome in Cell Biology Using Natural Products and Other Small Molecule Proteasome Inhibitors

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Introduction

In the era of systemic proteomics, temporal and spatial control of protein functions has become very important in the investigation of complex biological processes in vivo. While traditional genetic manipulations have provided a powerful tool to study protein function, these applications are limited by the possibility that some mutant phenotypes may be due to compensatory responses that occur during development. In addition, gene knockout models that are embryonic lethal are not amenable to study disease processes that occur in the adult animal. Moreover, the inhibition of the target gene's function is often irreversible, and thus, the desired biological effect(s) cannot be readily regulated. This makes it difficult to dissect the precise role of proteins in complex signaling pathways. Recently, small interfering RNA (siRNA[a]) has been widely used to modulate protein function at the RNA level.[1] However, this technique offers limited temporal control of gene expression. Difficulties with the nonspecificity and delivery of siRNAs have also been major concerns. The use of small-molecule probes is one way to complement these genetic approaches. Most biologically active small molecules including natural products exert their activities via inhibition of specific biological processes. In comparison to the classical genetic approach, this small molecule approach easily affords more temporal and spatial control of targeted biological events. A small molecule approach, being complementary to the classical genetic approach, is thus fittingly dubbed "chemical genetics".[2] Although many areas of biology have benefited from the chemical genetics approach, few have been more broadly and significantly impacted than the biology and biochemistry of the proteasome.

Proteasome Inhibitors: Natural Products and Other Small Molecules

The development of bortezomib (**1**),[3] the first proteasome inhibitor approved by U.S. Food and Drug Administration (FDA), validated the proteasome as an antitumor target. Before **1**, other proteasome inhibitors proved useful as powerful tools in the investigation of many important cellular processes regulated by the ubiquitin−proteasome pathway. This pathway serves as the principal conduit for protein turnover in eukaryotic cells.[4] By use of a 76 amino acid polypeptide, ubiquitin, a protein can be targeted for destruction by the 26S proteasome. A chain of ubiquitin molecules is coupled to the protein, and it is this conjugated system that is recognized by the proteasome. The 26S proteasome is composed of the multisubunit 20S catalytic core and 19S regulatory complexes, which assist in binding and unfolding ubiquitinated protein substrates.

Although originally dismissed as a "garbage disposal", in the past decade the proteasome has been recognized as a central player in the regulation of many important biological processes largely because of the development and use of highly specific proteasome inhibitors as molecular probes. While inhibitors of cysteine and serine proteases were initially used as proteasome inhibitors, more refined and specific proteasome inhibitors have become available via synthetic efforts, providing chemical genetic tools to study proteasome biology.[5] Synthetic proteasome inhibitors (see Chart 1A), most notably peptide aldehydes **2** and **3**[6] and vinylsulfone NLVS (**4**),[7] are cell-permeable and potent chymotrypsin-like activity inhibitors of the 20S proteasome still widely used in the study of proteasome biology. On the other hand, biologically active natural products, which are rich in chemical diversity, have also provided highly potent proteasome inhibitors with unusual chemical structures and pharmacophores (see Chart 1B). Lactacystin (**5**)[8] and epoxomicin (**6**)[9] are the best known examples of natural product proteasome inhibitors and are also currently widely used in proteasome research. These natural products, along with synthetic proteasome inhibitors, have greatly contributed to our understanding of proteasome biology.

Cell Proliferation

Many natural product proteasome inhibitors have been isolated on the basis of their antibiotic activity, proteasome inhibitory activity, antitumor activity, or induction of neurite outgrowth.[8–10] They were later found to induce apoptosis or inhibit cell proliferation because of activity toward the proteasome. Similarly, synthetic approaches have aimed to produce proteasome modulators having potent inhibitory activity against the chymotrypsin-like activity of the proteasome and thus antitumor activity.[11] Given that the proteasome plays an important role in cell survival and proliferation, it is not surprising that proteasome inhibitors have been used as molecular probes to investigate the role of the proteasome in cell cycle progression and apoptosis. Initially, the hypothesis that the proteasome regulated cell cycle progression was difficult to test because the only available proteasome inhibitors, such as compounds **2** and **3**, interfered with the activity of nonproteasomal proteases, such as calpains and lysosomal cathepsins.[12] However, the discovery and use of a highly specific proteasome

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[a] Abbreviations: siRNA, small interfering RNA; CDK, cyclin-dependent kinase; MDM2, murine double minute 2; Bid, BH3-interacting-domain death agonist; Bik, Bcl-2 interacting killer; Bak, Bcl-2 antagonist killer; Bax, Bcl-2 associated X protein; E6-AP, E6-associated protein; NF-κB, nuclear factor-κB; IκB, inhibitor of NF-kB; SCF, Skp1-Cullin-F-box; BMP, bone morphogenetic protein; TGFβ, transforming growth factor β; Smad, SMA/MAD related; Smurf1, Smad ubiquitin regulatory factor 1; Hect, Homologous to E6-associated protein C-terminus; Cbfa1, core-binding factor α1.

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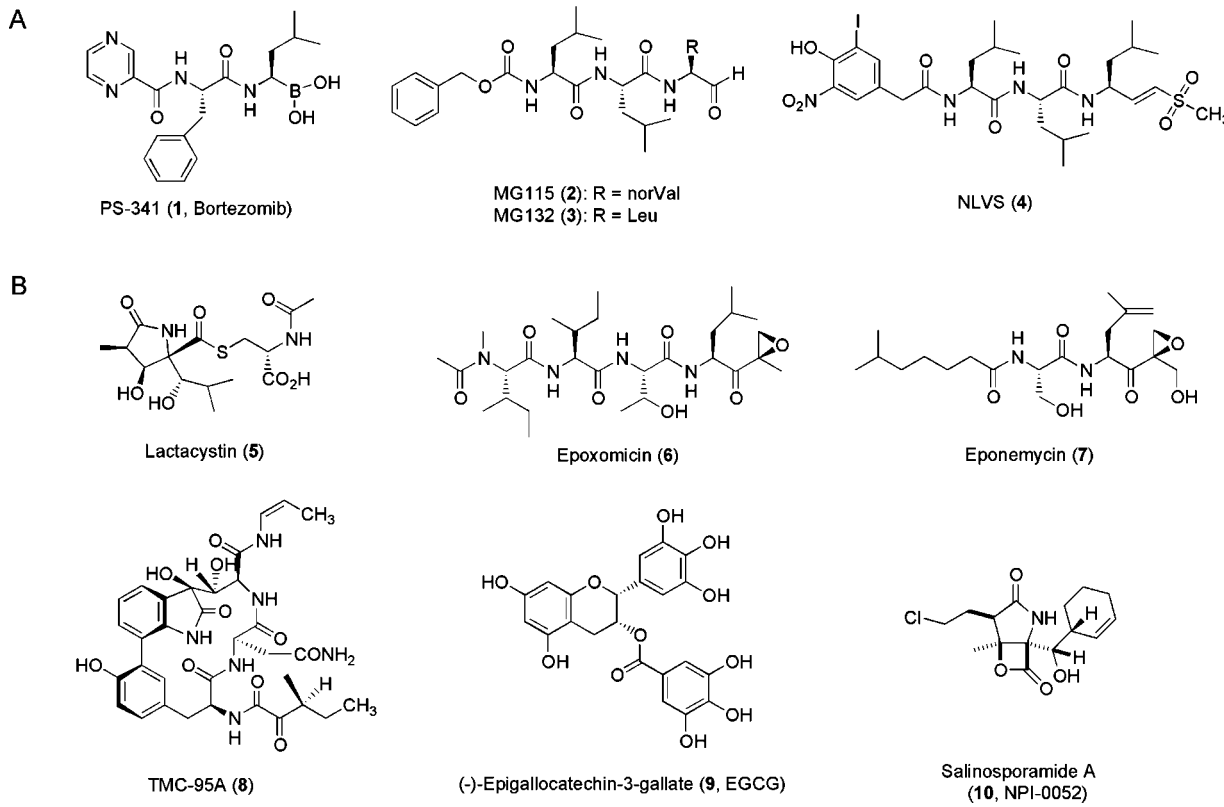
Chart 1. (A) Synthetic and (B) Natural Product Proteasome Inhibitors

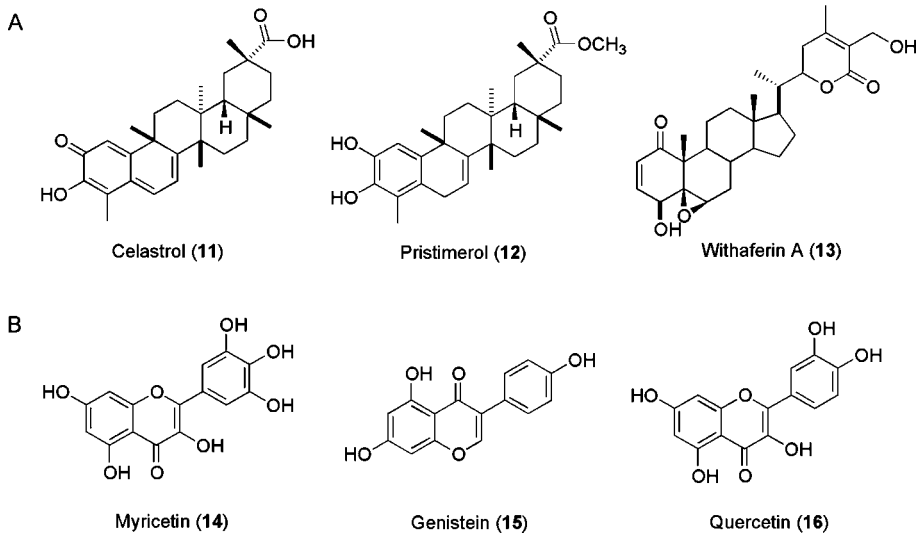


inhibitor **5** demonstrated that the proteasome is a major regulatory complex required for cell cycle progression.[13] It has been known for some time that orderly progression through the cell cycle requires programmed and periodic expression of certain proteins such as cyclins.[14] Cyclins were the first proteins that were shown to vary in expression level during the cell cycle progression. The oscillation of cyclins was found to be due to their tightly regulated degradation of cyclins. The use of proteasome inhibitors as molecular probes revealed that the proteasome is involved in the coordination of cyclin degradation. For instance, when proteasome inhibitor **2** or **5** was added to proliferating cells, the oscillation of cyclins was stopped, elegantly demonstrating that this oscillation was due to the regulated degradation mediated by the ubiquitin−proteasome system.[15] **2** and **5** also induced the G_0/G_1 cell-cycle arrest through the accumulation of the cyclin-dependent kinase (CDK) inhibitor p27, demonstrating that degradation of p27 is required for cells to proceed into S phase. In addition, these proteasome inhibitors increased protein levels of another CDK inhibitor, p21, as well as other cyclins A, B, D, and E, while also affecting transcription factor E2F and Rb.[16]

Proteasome inhibitors have proven equally useful in the study of the tumor suppressor protein p53.[17] This transcription factor is a key regulator in an intricate network of proteins with diverse functions that include sensing different stress signals.[18] The activation of p53 in response to such signals typically results in apoptosis or cell-cycle arrest, thereby maintaining the integrity of neighboring normal cells. Since the up-regulation of p53 can result in such a drastic cellular consequences as apoptosis, the basal level of p53 must be tightly regulated. One of the major regulators of the p53 protein level is the murine double minute 2 (MDM2) protein. Researchers found that by inducing the accumulation of p53 using the natural product proteasome inhibitor **5**, the degradation of p53 is dependent on the ubiquitin−proteasome system.[17] Further studies revealed that the MDM2 functions as a p53-specific E3 ubiquitin ligase and thus triggers the ubiquitination and subsequent proteasomal degradation of p53.[19] Currently, accumulation of p53 is widely used as a prototypical marker of the activity of proteasome inhibitors in cells. In addition, taking advantage of their ability to induce apoptosis in a p53-dependent manner, proteasome inhibitors have been employed to probe the role of other pro- and antiapoptotic proteins in p53-dependent apoptotic signaling pathways.

It appears that proteasome inhibitors also induce apoptosis via p53-independent pathways.[20] Specifically, proteasome inhibitors can cause apoptosis in cell lines lacking p53 by directly activating proapoptotic proteins, thus helping to elucidate p53-independent apoptotic pathways. For instance, apoptosis caused by compound **2** in p53-deficient cells has been attributed to the accumulation of the proapoptotic Bcl-2 family of proteins, which are normally degraded by the ubiquitin−proteasome system.[21] The Bcl-2 protein family is composed of both antiapoptotic and proapoptotic proteins and plays a pivotal role in controlling cell survival by regulating mitochondrial-initiated apoptosis. By use of proteasome inhibitors, it has been easily observed that the proteasome modulates Bcl-2 mediated apoptosis primarily by affecting the half-life of two of the "BH3-only" proteins, BH3-interacting-domain death agonist (Bid) and Bcl-2 interacting killer (Bik).[22] One notable example is that in leukemia cells, apoptosis induced by proteasome inhibitors was accompanied by the accumulation of Bik in the mitochondria,[23] clearly indicating a major function of the proteasome in regulating Bik in cells. This small molecule approach was further corroborated by a conventional genetic approach in which Bik overexpression was sufficient to trigger apoptosis in these cells. Similarly, Bid was stabilized by proteasome inhibitors, causing it to release cytochrome *c* and subsequently triggering apoptosis in cells.[24] The death-promoting Bcl-2 proteins, Bcl-2 antagonist killer (Bak) and Bcl-2 associated X protein (Bax), also seem to be

Chart 2. (A) Terpenoids and (B) Dietary Flavonoids Having Proteasome Inhibitory Activities



activated by proteasome inhibitors,[25] indicating that the proteasome plays a role in regulating these death proteins.

In addition, proteasome inhibitors induced apoptosis of differentiating human epidermal keratinocyte cells that express the oncoprotein human papillomavirus E6.[26] This oncoprotein can normally inhibit apoptosis in differentiating keratinocytes by promoting degradation of the proapoptotic protein Bak, which is highly expressed in keratinocytes. Specifically, inhibition of apoptosis in cells expressing E6 is caused by the formation of E6-associated protein (E6-AP), an E3 ubiquitin-ligase, which interacts with Bak and induces its degradation. Therefore, the treatment of proteasome inhibitors in these cells induces the accumulation of Bak and thus results in apoptosis. In addition to the apoptotic proteins mentioned above, numerous regulatory molecules that are involved in programmed cell death have been identified as substrates of the proteasome.[22] Since the temporal regulation of the functions of proteins associated with apoptosis is often impossible with genetic manipulations, small-molecule proteasome modulators will continue playing important roles in the unraveling of complex apoptotic signaling pathways.

Recently, a number of chemopreventive or antitumor dietary flavonoids and triterpenoids (compounds **11**–**16**, Chart 2) have been reported to possess proteasomal inhibitory activity.[27] Although these natural products have been shown to primarily inhibit the chymotrypsin-like activity of the proteasome in vitro, it is still not clear whether the proteasome mediates the pharmacological activities of these natural products in vivo. Therefore, mode of action studies on these biologically active natural products that possess proteasomal inhibitory activity will undoubtedly help to dissect signaling pathways associated with their chemopreventive or antitumor activities.

Inflammatory Responses

The use of proteasome inhibitors as molecular probes has also impacted research into nuclear factor-κB (NF-κB)-mediated inflammatory signaling pathways. NF-κB is a heterodimeric transcription factor composed of two subunits, p50 and p65, and is responsible for activating the transcription of a variety of genes and adhesion molecules involved in cellular inflammatory responses.[28] NF-κB is normally sequestered in the cytoplasm in an inactive form because of its binding to the inhibitory protein, inhibitor of NF-κB (IκB). Activation of NF-κB is achieved by the ubiquitination and subsequent degradation of IκB by the proteasome, which is caused by either cytokines or intracellular stress signals that result in phosphorylation of IκB.

A chemical genetic approach, in combination with classical yeast genetic studies, has again proven useful in dissecting complex NF-κB-activating mechanisms. For example, Palombella et al. showed in a yeast model system that the proteasome is required for the limited processing of p105, a p50 precursor protein, to release the p50.[6] Once processed, the newly generated p50, together with p65, forms the transcriptionally active heterodimeric NF-κB. Complementary to these yeast genetic studies, peptide aldehyde proteasome inhibitors **2** and **3** were used to demonstrate that the functionally active proteasome is required for the processing of p105 in intact mammalian cells. In fact, IκB was the first proteasome substrate identified in inflammatory signaling pathways by the use of proteasome inhibitors as molecular probes. Normally, in response to inflammatory stimuli, the cytosolic IκB proteins complexed with NF-κB are rapidly phosphorylated and degraded to trigger inflammatory signaling cascades. However, incubation with compound **3** (or **5**) results in inhibition of inflammatory responses via stabilization of both IκB and phosphorylated IκB caused by the blockade of IκB degradation, clearly demonstrating that the proteasome is a major regulator of inflammatory signaling pathways. Later, a detailed investigation of IκB degradation using proteasome inhibitors led to the characterization of interactions between phosphorylated IκB and the E3 ubiquitin ligase complex Skp1-Cullin-F-box (SCF)$^{\beta\text{-TRCP}}$.[28]

Natural product and synthetic proteasome inhibitors have also provided a powerful tool in investigating the roles of NF-κB in the development of drug-resistant tumors, where increased NF-κB activation has been repeatedly observed.[29] For instance, a combination of both conventional genetic and chemical genetic approaches has allowed for the precise modulation of NF-κB activation in transformed cells, which is important to investigate signaling pathways in both in vivo and in vitro models. First, a conventional genetic approach was employed to prepare a mutant form of IκB that cannot be ubiquitinated (i.e., serines that are recognized by ubiquitinating enzymes when phosphorylated were mutated to alanines), which restores the drug sensitivity in a cell-based system.[29b] Although this genetic manipulation indicates the potential role of NF-κB in drug-resistant cell models, this may not "truly" reflect the role of

NF-κB in vivo. By demonstration of the power of chemical genetics, proteasome inhibitors have easily enabled the corroboration of these in vitro findings with an in vivo model. For example, in an in vivo animal model, combination of a conventional cancer drug with the boronic acid-based proteasome inhibitor **1** resulted in a significantly higher level of tumor growth inhibition compared to treatment with **1** alone or the conventional cancer drug alone,[30] confirming in vitro findings. It appears that development of tumors resistant to conventional cancer drugs is due to the antiapoptotic role of activated NF-κB in some cancer cell models. However, it remains possible that signaling events other than NF-κB inactivation may also play important roles in inducing apoptosis of cells treated with proteasome inhibitors.

Bone Growth

Another research area that has tremendously benefited from the use of proteasome inhibitors is the exploration of bone morphogenetic protein (BMP)-regulated signaling pathways. BMPs are multifunctional growth factors that belong to the transforming growth factor β (TGFβ) superfamily.[31] BMP activity was first discovered in the 1960s,[32] and the protein was purified in the 1980s.[33] Recent genetic studies have shown that BMPs play critical roles in bone and cartilage development, promoting postnatal bone formation by stimulating the proliferation and differentiation of osteoblasts.[34] Thus, it has been suggested that non-union of the bone and delayed healing from bone resorption may be the result of decreased levels of BMP activity. Activation of BMP receptors has been shown to initiate phosphorylation of the downstream effector proteins, known as receptor-regulated Smads, leading to activation of BMP signal transduction cascades.[31] The BMP receptor-regulated Smads form a hetero-oligomeric complex with a common mediator SMA/MAD related (Smad) protein, which translocates into the nucleus and regulates target gene transcription. Extracellular BMP antagonists, such as a protein called Noggin,[35] are shown to block postnatal bone growth and bone formation through inhibition of BMP receptor binding to Smads. It is also shown that the BMP signaling cascade is negatively regulated by certain inhibitory Smads.[31] However, the regulatory mechanism of BMP signaling via these inhibitory Smads is not fully understood.

Recently, it has been observed that natural product proteasome inhibitor **6** and other proteasome inhibitors increase bone formation rates in in vitro and in vivo models.[36] It is suggested that bone formation may be promoted by proteasome inhibitor-induced accumulation of BMP-2 and Smads 1 and 5, the two Smad proteins having been shown to play an important role in osteoblast differentiation.[37] Furthermore, the Smad family proteins have been shown to interact with various components of the 26S proteasome system.[38] On the basis of these observations, it has been suggested that the proteasome may play a role in the regulation of Smad signaling pathways. Additional studies demonstrated that the E3 ubiquitin ligase Smad ubiquitin regulatory factor 1 (Smurf1), a member of the homologous to E6-associated protein C-terminus (Hect) domain family, induces ubiquitination and subsequent degradation of the Smad proteins.[38] Smurf1 has been also shown to play a key role in the regulation of osteoblast differentiation and bone formation in vivo. For example, in transgenic mice overexpressing Smurf1, bone formation was significantly reduced during postnatal life.[38] The negative regulation of bone formation by Smurf1 was further evidenced by proteasome inhibitors in vivo. Systemic treatment of peptide aldehydes or natural product proteasome inhibitor **6** induced significant new bone formation,[38] antagonizing the inhibitory action of Smurf1. In intact and ovariectomized mice, these proteasome inhibitors stimulated bone formation via accumulation of Smad1 and core-binding factor alpha 1 (Cbfa1),[36] a bone-specific transcription factor required for BMP-2 signaling, demonstrating the proteasomal regulation of Smad1 and Cbfa functions in osteoblast differentiation. As shown by these examples, proteasome inhibitors are useful tools for the exploration of the role that the proteasome plays in bone growth and will continue to help to elucidate a detailed description of BMP signaling events during bone differentiation.

Conclusion: Future Directions

The discovery that several antitumor natural products exert their action via proteasome inhibition has provided a rationale to develop proteasome inhibitor drugs for cancer treatment, culminating in the FDA approval of bortezomib for the treatment of multiple myeloma.[3] Currently, several proteasome inhibitors derived from natural products are being investigated for their antitumor activity in clinical trials. Given the ability of proteasome inhibitors to control proteasome function in vitro and in vivo, they will continue to be powerful tools in the investigation of biological events that are regulated by the proteasome−ubiquitin system in cells.

A current question under investigation concerns the role of the different catalytic subunits of the 26S proteasome in complex signaling pathways. Earlier studies with chymotrypsin-like activity-specific proteasome inhibitors have led to the conclusion that among the major proteolytic activities of proteasome, the chymotrypsin-like activity is primarily responsible for the proteasome's regulatory functions in vivo and in vitro.[39] Similarly, most of the antitumor proteasome inhibitors are directed against chymotrypsin-like activity. For example, using model proteins, researchers demonstrated that simultaneous inhibition of the chymotrypsin-like and the caspase- or trypsin-like activities is needed to reduce degradation by greater 50%.[39b] Concerning this uncertainty regarding the contributions of the different proteasomal catalytic activities, future studies will undoubtedly include the development of more refined inhibitors that target specific proteolytic activities in order to dissect their contribution more precisely.

In addition to the constitutive proteasome described above, the immunoproteasome, an alternative form of the constitutive proteasome, is an emerging target for the chemical genetic approach. Despite recent advances implicating the role of the immunoproteasome in certain pathological disorders[40] such as neurodegenerative diseases and cancer, any physiological role of the immunoproteasome beyond MHC class I antigen presentation is largely unknown. The major hurdle limiting a more complete understanding of immunoproteasome biology is the lack of appropriate molecular probes that selectively target the immunoproteasome's catalytic subunits. Therefore, natural products or synthetic small molecules that selectively target the immunoproteasome subunits will provide a powerful chemical genetic tool to investigate the physiological roles of the immunoproteasome subunits. Recently, Ho et al. have developed a "first generation" of immunoproteasome-specific inhibitors designed on the basis of the natural product proteasome inhibitor eponemycin (**7**).[40e] Considering the progress made toward an understanding of the proteasome biology using small molecules in the past few decades, the coming years will be exciting times in the field of proteasome biology as these novel immunoproteasome inhibitors become available.

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Biographies

Kyung Bo Kim received his Ph.D. in 1997 from The Ohio State University under the supervision of Prof. Edward Behrman. After postdoctoral studies with Prof. Craig Crews at Yale University, he joined the University of Kentucky in 2002, where he is currently Assistant Professor of Pharmaceutical Sciences. His research interests focus on the development of novel classes of proteasome modulators and other biologically active small molecules that can be used as molecular probes as well as drug leads.

Craig M. Crews received his Ph.D. from Harvard University in 1993, working in the laboratory of Prof. Ray Erikson. After completing his postdoctoral training with Stuart Schreiber at Harvard University, he joined the faculty at Yale University where he is currently Professor of Molecular, Cellular, and Developmental Biology as well as of Chemistry and Pharmacology. His research interests lie at the interface of chemistry and biology. In particular, his laboratory uses biologically active synthetic and natural products to explore a wide array of complex biological processes such as regeneration and inflammation and to identify and validate novel drug targets.

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