CCR frontiers IN SCIENCE

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From the Director

The mission of the Center for Cancer Research (CCR) is to conduct outstanding, cutting-edge basic and clinical research on cancer and to translate these discoveries into treatment and prevention that will reduce the burden of cancer in humans. Rather than separate activities of basic and clinical research, we now have closely linked the basic researcher with the clinical investigator. In addition to research, a key component of our mission is to provide training to the future leaders in cancer research.

The creation of the CCR takes advantage of the breadth of our researchers. We have a broad spectrum of scientists doing cutting-edge research in multiple areas. We have the ability to go from basic research to pre-clinical models to human trials very quickly and to develop new technologies that can be used in translational research. Furthermore, we can leverage the expertise and the infrastructure in the CCR with academic partners, other government laboratories, and pharmaceutical companies. We are in the process of developing the infrastructure to make this possible.

The overall goal is for the CCR to have a highly interactive, interdisciplinary group of researchers who have access to both technology and clinical investigations, while maintaining the foundation of investigator-initiated, independent research. We want to create opportunities for interaction by building the infrastructure that allows scientists to engage other researchers as interdisciplinary teams.

With the creation of the CCR, I think we will see an increase in communication, interaction, and translational research. To go from bench to bedside requires a capacity not available to most individual investigators. Previously, there were no roads built between basic and clinical research; basic researchers had to forge their own roads one by one. Now the institutional infrastructure is established. Equally important is the opportunity for clinical investigators to engage basic researchers in probing important clinical observations.

We are discussing how to most efficiently organize the CCR to open access to new technologies and clinical resources, and how to challenge the CCR researchers to become increasingly involved in translational research. We hope it becomes common for interdisciplinary groups to go from the bedside to the bench and from the bench to the bedside.

We are also building partnerships to encourage interdisciplinary research. We are moving from a culture of the individual scientist working alone within his or her own laboratory to scientists working within the community of the Intramural Program and within the network of the cancer research community—across the private sector, government, and academia. The CCR will serve as a model to facilitate translational research.

We hope that the CCR can be a resource for generating ideas that will help guide the direction of the NCI—to be an experimental laboratory for new ideas, new technologies, and new approaches to interdisciplinary research. We think that the CCR and the Intramural Program are important resources for the NCI.

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CCR frontiers IN SCIENCE

Mission

CCR Frontiers in Science was developed to foster scientific communication within the Center for Cancer Research (CCR) by: 1) promoting awareness of cutting-edge scientific results coming from the Center; 2) fostering scientific collaborations; 3) presenting information on COREs, technologies, and other scientific resources; and 4) providing helpful administrative news briefs with links to corresponding Web sites. Your contributions and comments are welcome. Please send proposals for articles, new ideas, and suggestions to the editor, Sue Fox, by email at smfox@mail.ncifcrf.gov or by telephone at 301-846-1923.

If you have scientific news of interest to the CCR community, please contact one of the Scientific Advisory members responsible for your area or Sue Fox at smfox@mail.ncifcrf.gov.

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Clinical Trials Highlights

01-C-0011
Robert Kreitman, M.D.

A Phase I Study of SS1(dsFv)-PE38 (SS1P) Anti-Mesothelin Immunotoxin in Advanced Malignancies: Continuous Infusion x10 days

This study specifically tests the efficacy of the recombinant immunotoxin SS1(dsFv)-PE38 (SS1P) against tumors expressing mesothelin, such as mesothelioma; non-mucinous epithelial ovarian cancer; and squamous cell carcinoma of the lung, head and neck, and cervix. SS1P is composed of an anti-mesothelin variable fragment fused to truncated Pseudomonas exotoxin. Patients must have recurrent, unresectable disease after appropriate, definitive therapy.

01-C-0104
Edward Sausville, M.D.

A Phase I Study of Concomitant Therapy with Proteasome Inhibitor PS-341 and Radiation in Patients with Recurrent or Metastatic Squamous Cell Carcinoma of the Head and Neck

The ubiquitin-proteasome pathway regulates the degradation of important regulatory proteins and transcription factors that control cell cycle and cell death. Proteasome inhibition can block different phases of the cell cycle as well as expression of genes that prevent cell death induced by radiation or other cytotoxic therapeutic agents. In pre-clinical studies, the proteasome inhibitor PS-341 has demonstrated cytotoxic, radiosensitizing, and anti-tumor activity against squamous cell carcinomas of the head and neck. The primary objective of this study is to determine the maximum tolerated dose of PS-341 to be given concomitant with radiation to patients with recurrent or metastatic squamous cell carcinoma of the head and neck.

00-C-0121
Jonathan Wigginton, M.D.

A Phase I Investigation of IL-12/Pulse IL-2 in Adults with Advanced Solid Tumors

Recombinant IL-12 is a recently described cytokine possessing potent anti-tumor activity. In animal models, IL-12 has shown significant therapeutic activity, both as a tumor vaccine adjuvant and when administered systemically alone or in combination with other cytokines such as IL-2. This study is the first to test the IL-12—IL-2 combination in humans and will assess its maximum tolerated dose, its pharmacokinetics, and its immunoregulatory and anti-tumor effects when administered intravenously to adults with various advanced and refractory solid tumors.

00-C-0212
Sandra Swain, M.D.

A Phase I/II Trial of Docetaxel Followed by Infusional Flavopiridol Over 72 Hours in Patients with Previously Treated Locally Advanced or Metastatic Breast Cancer

In this study, docetaxel, a known taxane used in the treatment of breast cancer, is administered then followed by a continuous infusion of flavopiridol, a cyclin-dependent kinase inhibitor. The Phase I study will determine the maximum tolerated dose of the combination, and the Phase II portion will determine the response rate. Translational endpoints include immunohistochemical measurement, both pre- and post-treatment, of cyclin D1, p53, Bcl-2, ki67-mib1, mitosis, and apoptosis from paired tumor samples. Measurements of these parameters in buccal mucosa and in normal surrogate tissue will also be evaluated. PET scans will be performed to assess the ability to measure tumor response.

00-C-0218
David Bartlett, M.D.

A Phase II Trial of Combined Intraperitoneal Gemcitabine, Intravenous Gemcitabine, Radiotherapy, and Surgery for Advanced Adenocarcinoma of the Pancreas

This study evaluates the response of locally unresectable adenocarcinoma of the pancreas, alone or in association with small-volume, resectable metastases of the peritoneal cavity. Patients will receive two cycles of weekly intraperitoneal gemcitabine, each administered over 24 hours, followed by intravenous (IV) gemcitabine combined with radiotherapy in a standard care manner over six weeks. Patients achieving complete extrapancreatic resection of disease will undergo two repeated cycles of weekly intreperitoneal gemcitabine, and all patients will then receive a combination of weekly IV gemcitabine and prolonged IV infusion of 5-FU for up to 6 months. Patients will be assessed for toxicity, tumor response, progression-free survival, and overall survival. Pharmacokinetics of intraperitoneal gemitabine will be studied, and tumor mRNA levels will be examined for patterns of gene expression that may predict prognosis and chemotherapy response.

00-C-0154
William Dahut, M.D.

A Randomized Phase II Study of a PSA-based Vaccine in Patients with Localized Prostate Cancer Receiving Standard Radiotherapy

This trial evaluates the immunologic effects of a vaccination regimen on HLA-A2-positive prostate cancer patients. Eligible patients will have localized prostate cancer and be willing to undergo definitive local radiotherapy. Thirty patients will be randomized in a 2:1 ratio into two groups; patients in the vaccine arm will receive vaccination before, during, and after primary standard radiotherapy. The vaccine regimen will consist of a recombinant vaccinia virus that expresses the prostate-specific antigen gene (rV-PSA) mixed with a recombinant vaccinia virus that expresses the B7.1 costimulatory molecule (rV-B7.1), followed by sequential vaccinations with recombinant fowlpox virus containing the PSA gene (rF-PSA). All patients on the vaccine arm will, in addition, receive sargramostim and aldesleukin as part of their vaccination schedule.

99-C-0051
Surgery Branch
Studies of Cancer Vaccines

Center for Cancer Research is the Coordinating Center

The Surgery Branch has initiated several studies evaluating the role of peptide vaccines for the treatment of patients with metastatic melanoma. The genes encoding several melanoma antigens have been identified, and the immunodominant peptides from these encoded proteins are being used to immunize patients with metastatic melanoma. The immunogenicity of these peptides have been enhanced by modifications at positions that anchor the peptides to surface major histocompatibility complex (MHC) molecules. Immunization with these peptides results in the development of strong anti-tumor T-cell responses and, when administered in conjunction with IL-2, appears to increase the objective response rate of patients with metastatic melanoma compared with IL-2 treatment alone. A prospective randomized trial is now being conducted at 13 institutions to compare the combination of IL-2 and peptide with IL-2 alone. In the Surgery Branch, NCI, current protocols are evaluating the combined administration of both class I- and class II-restricted peptides as well as the adoptive transfer of cloned T-cells with anti-tumor reactivity.

01-C-0124
Edward Sausville, M.D.

A Phase I Study of MS-275 in Patients with Solid Tumors and Lymphomas Refractory to Standard Therapy

Histone deacetylases (HDACs) are critically important in the regulation of gene expression and in the field of target-specific anticancer drug development. Several HDAC inhibitors have been shown to have a wide range of effects, including gene activation, cellular differentiation, cell growth arrest, and apoptosis. The HDAC inhibitor MS-275, a benzamide derivative, has demonstrated potent and unique cytotoxicity and anticancer activity *in vitro* in human tumor cell lines and, more importantly, *in vitro* in human tumor xenografts. The purpose of this MS-275 Phase I trial is to explore a potential novel chemotherapeutic agent for cancers that are insensitive to traditional anti-tumor agents. Patients with solid tumors and lymphomas refractory to standard therapy, or those patients for whom there is no known standard therapy that is potentially curative or capable of extending life expectancy, will be evaluated for study eligibility without consideration of race, ethnic origin, gender, or sexual orientation. Because of the absence of experience in using MS-275 in humans, children, pregnant women, and HIV-infected persons are excluded from this study. This study will evaluate the pharmacokinetics and pharmacodynamics of MS-275 in humans and will determine the optimal dose and schedule that is suitable for clinical use. Also, this trial will analyze MS-275-induced changes in HDAC activity and gene expression in relation to its anticancer activity.

Protein Goes to Pieces Inside Clp Joint!

Ortega J, Singh SK, Maurizi MR, and Steven AC. Visualization of substrate binding and translocation by the ATP-dependent protease. *Mol Cell* 6: 1515-21, 2000.

Singh SK, Grimaud R, Hoskins JR, Wickner S, and Maurizi MR. Unfolding and internalization of proteins by ClpXP and ClpAP. *Proc Natl Acad Sci USA* 97: 8898-903, 2000.

The degradation of proteins (proteolysis) is an essential intracellular process involved in normal and abnormal development, cell growth, stress responses, and other signaling pathways. Proteolysis is also required for protein quality control and antigen presentation. How do intracellular proteases degrade appropriate substrates without damaging normal proteins? This question is particularly pertinent for non-compartmentalized soluble proteases that must encounter a great many proteins whose degradation would cause irreparable harm to the cell.

One fundamental principle underlying control of proteolysis is reflected in the unique molecular architecture of the ATP-dependent proteases responsible for most intracellular protein degradation. In the known ATP-dependent proteases, such as the 26S proteasomes, the Clp and Lon proteases, and FtsH, the protein degrading function is self-compartmented. The proteolytic core is assembled so that the active sites are sequestered within an aqueous chamber formed by the joining of two rings, each composed of six or seven protease subunits. The chamber is closed on all sides and accessible only through a single, narrow axial channel in each ring, rendering the core virtually inactive against proteins and peptides of more than about 10 amino acids.

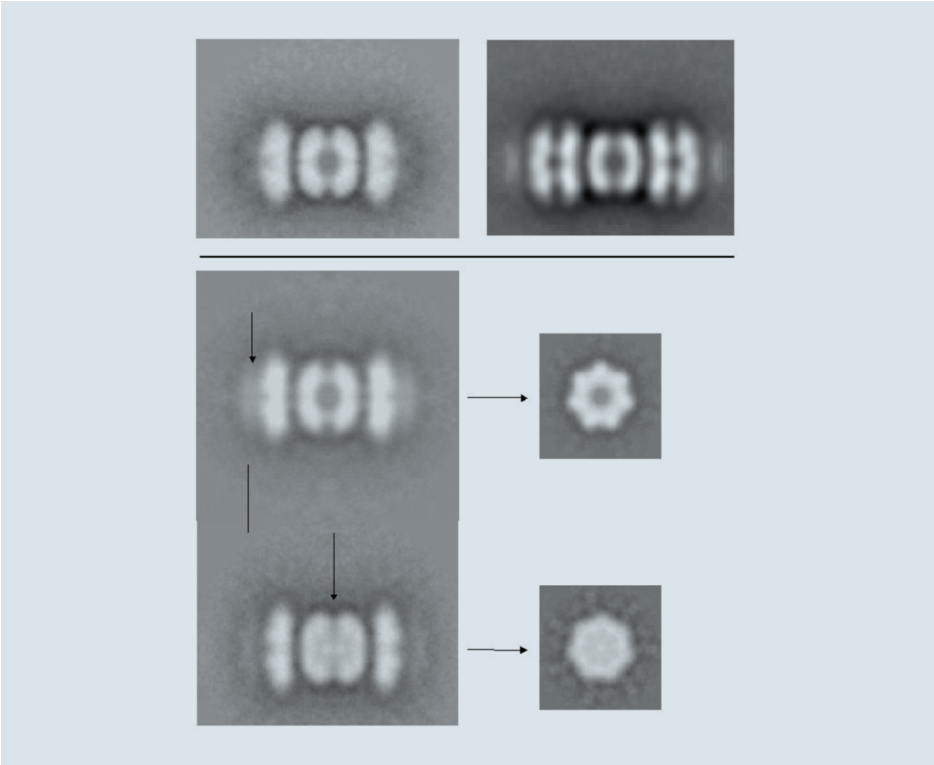

Figure 1. ATP-dependent proteases, ClpXP and ClpAP, bind substrates at sites located on the apical surfaces of barrel-like complexes and then must unfold the substrates from one end and shunt them through narrow axial channels into an interior degradative chamber.

Effective use of the core for proteolysis depends on multifaceted regulatory complexes that are tightly associated with it. In some cases, such as Lon and FtsH, the regulatory domains are independently folded parts of the same polypeptide chain. The regulatory particles range from the very complex (as many as 19 different subunits in the regulator of the mammalian proteasome) to the simple (6 identical subunits in regulators of the Clp protease family). Included in all the regulatory complexes is a ring of ATPases belonging to the AAA^+ superfamily, ATP-powered enzymes that catalyze protein unfolding and disassembly. The ATPases make direct contact with the protease and are the motor in the machine, driving the process by which even the simplest assemblies somehow enable folded proteins to be taken into the proteolytic chamber efficiently and selectively.

We have employed a combination of biochemistry and single-particle electron microscopy to obtain a better understanding of the mechanism of ATP-dependent proteolysis. Our latest results (Ortega et al., *Mol Cell* 6: 1515-21, 2000) provide striking images of the starting and ending points of the process by which proteins are captured and degraded and confirm, to a large degree, our basic model for the action of ATP-dependent proteases. Our studies have focused on *Escherichia coli* ClpXP and ClpAP, which belong to the Clp family of ATP-dependent proteases. In Clp proteases, a single proteolytic core particle, ClpP, can combine with either of two ATPases, ClpA or ClpX, to form separate ATP-dependent proteases with

distinct substrate specificities. When ClpXP or ClpAP complexes are viewed lying on their sides, the stacked rings of ClpP and ClpX or ClpP and ClpA are seen on edge, and the parallel bands give the complex a barrel-like appearance (Figure 1). The stain used for imaging penetrates the interior chambers, rendering them electron dense, so that the walls of the chambers are not visible from the side. In top views of ClpP, the thickness of the chamber walls is more obvious. Interior chambers or vestibules are also present in ClpA and are formed by the junction of the ClpA or ClpX and ClpP. Protein within a chamber reduces stain penetration, allowing us to identify those complexes that have taken up protein substrate.

Our results show the starting and ending points of the process by which proteins are captured and degraded.

One goal of our investigation was to visualize substrate proteins bound to the protease complex at different stages of the reaction. To identify the initial site of substrate binding, we obtained images of substrate/ClpXP complexes trapped in ATPγS, which showed the substrate, λO, bound to the outer ring surface of ClpX (Figure 1). We then added ATP, which we knew from biochemical studies with active enzyme allowed degradation of the substrate without disassembly of the complex. For microscopy, we avoided substrate degradation by using inactivated ClpP. Thus, after ATP addition, we saw the substrate disappear from the outer surface but reappear in the center of ClpP. To confirm translocation, we separated ClpX and ClpP and obtained top views of ClpP, which clearly showed protein accumulation in the central chamber. These images provided graphic proof that proteins are transferred to the center of ClpP for degradation and, moreover, showed that simultaneous peptide bond cleavage is not required for protein translocation.

The work of translocation falls to the ATPase components. Clp ATPases have protein unfolding activity and belong to an extended family that includes some autonomous chaperones. Specific sequences located near the N- or C-terminus of a substrate provide recognition motifs, allowing interaction with ClpX or ClpA, probably to a site involving some part of the axial channel. Once bound, the protein is unfolded in an ATP-dependent process (Singh et al., *Proc Natl Acad Sci USA* 97: 8898-903, 2000). In the absence of ClpP, the unfolded protein is released and can refold, but when ClpP is present, the protein is efficiently translocated into the degradation chamber. One current model is that unfolding and translocation occur simultaneously—proteins are essentially unstrung from one end, probably the end bearing the degradation motif. Local secondary structures would be broken in increments (an average of four to eight molecules of ATP are hydrolyzed per 10 amino acid peptide product generated) and thus no high-energy barriers would be faced in disrupting the substrate structure.

Domains attached to the ATPase or components associated with it provide substrate recognition sites and other regulatory features. Thus, overall, selectivity in intracellular proteolysis is accomplished by imposing different layers at which control can be exercised. First, recognition elements bind potential substrates, which are partially unfolded and handed off to a translocase, which then transfers the unfolded substrate to the degradation chamber. Multiple checkpoints may be present in the first stage—both in the nature of the recognition motif, which can be either intrinsic or, as in the case of poly-ubiquitin tagging, extrinsic—and in the partitioning of recognized proteins between release and hand-off. Checkpoints may also occur at the initial stages of unfolding, and such checkpoints can even be exploited, as in the case of limited processing, rather than complete degradation, of NFκB. Current studies are directed towards a better understanding of the initial recognition of substrates and how that differs from the binding interactions required to initiate unfolding; identifying the sequence and structural elements along the unfolded polypeptide chain that define the "translocation increments"; and finally describing the conformational changes in the "translocase" that enable it to hold a segment of substrate and move it through the channels.

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Translational Studies: Defining Strategies to Enhance the Potency of Cancer Vaccines

Grosenbach DW, Barrientos JC, Schlom J, and Hodge JW. Synergy of vaccine strategies to amplify antigen-specific immune responses and anti-tumor effects. *Cancer Res* 61: 4497-505, 2001.

Zhu M, Terasawa H, Gulley J, Panicali D, Arlen P, Schlom J, and Tsang KY. Enhanced activation of human T cells via avipox vector-mediated hyperexpression of a triad of costimulatory molecules in human dendritic cells. *Cancer Res* 61: 3725-34, 2001.

Tumor-associated antigens (TAAs) are, by definition, either weakly immunogenic or functionally nonimmunogenic in the tumor-bearing host. Thus, if vaccines are to be effective in cancer therapy, strategies must be developed to present TAAs to the immune system in a way that makes them more immunogenic. Investigators in the Laboratory of Tumor Immunology and Biology (LTIB), CCR, have developed several approaches to enhance the immunogenicity of TAAs and have developed animal models to evaluate these strategies as a step toward the translation of hypothesis-driven research to science-based clinical trials. Studies have demonstrated that two types of recombinant poxvirus vectors are very effective in enhancing antigen-specific T-cell responses: the replication-competent vaccinia (rV) and the replication-defective avipox viruses. The vaccine target for these studies is carcinoembryonic antigen (CEA), which is found overexpressed in the vast majority of human colorectal, pancreatic, and non-small-cell lung and breast cancers, in fetal gut, and, to a lesser extent, in normal colonic epithelium.

A CEA-transgenic mouse, which expresses CEA in a manner similar to that of humans, is being used to analyze vaccine strategies. Early studies demonstrated that while these mice are tolerant to vaccination with CEA protein in adjuvant, rV-CEA or avipox-CEA recombinants could indeed elicit CEA-specific T-cell responses. These findings led to Phase I clinical trials, in which it was demonstrated that both rV-CEA and avipox-CEA could elicit CEA-specific T-cell responses in patients with advanced carcinomas. Moreover, these T cells were shown to be capable of lysing tumors expressing CEA. An ELISPOT assay was developed to quantitate the magnitude of these T-cell responses prior to and after each vaccination. Concomitant experimental studies demonstrated that diversified prime and boost strategies (i.e., priming with rV-CEA and boosting with avipox-CEA) could induce more potent immune responses than strategies with either vector alone. These studies were then translated to a randomized Phase II trial in which patients with advanced CEA-expressing carcinomas who had failed conventional therapies received either rV-CEA (V) prime vaccination followed by three monthly avipox-CEA (A) booster vaccinations (i.e., the VAAA regimen) or the reciprocal AAAV regimen. Patients vaccinated with the VAAA regimen demonstrated the generation of significantly higher levels of CEA-specific T cells and longer survival than patients randomized to the AAAV regimen. There was also a statistically significant correlation between survival at more than 2 years post-vaccination and CEA-specific T-cell responses. These data were presented at the American Society of Clinical Oncologists (ASCO) 2001 Meeting by collaborator Dr. John L. Marshall (Lombardi Cancer Center, Georgetown University). These studies also demonstrated that patients could receive multiple vaccinations with avipox-CEA quantitatively and exhibit increasing CEA-specific T-cell responses.

These studies demonstrate that several novel strategies can synergize to enhance the potency of the immune responses to tumor antigens.

While the above clinical studies were ongoing, James Hodge, Ph.D., of the LTIB carried out a series of studies in which he demonstrated that the insertion of a transgene for one of several T-cell costimulatory molecules into poxvirus vectors, along with the transgene for a TAA, enhanced the potency of the immune response to the TAA. It is now known that for the initiation of a vigorous T-cell response, two signals are required. Signal 1 is mediated through a peptide/MHC complex and Signal 2 is mediated through a costimulatory molecule. Dr. Hodge demonstrated that the insertion of the transgenes for a TRiad of COstimulatory Molecules (B7-1, ICAM-1, and LFA-3; designated TRICOM) into these vectors, along with the TAA transgene, could markedly enhance T-cell responses to the TAA. Dr. Douglas Grosenbach and Dr. Hodge have now demonstrated the potency of CEA/TRICOM vector-based vaccination in a model in which CEA-transgenic mice are bearing well-established liver metastases expressing CEA. Tumor therapy effects were observed after vaccination with rV-CEA/TRICOM and boosts with avipox-CEA/TRICOM. These effects could not be achieved with other vaccine strategies.

Because T-cell costimulatory molecules are species specific, human TRICOM vectors were then designed

and constructed. Kwong Tsang, Ph.D., and colleagues (LTIB) have demonstrated that infection of peptide-pulsed human dendritic cells by TRICOM vectors greatly enhances the generation of antigen-specific T-cell responses. These studies, along with the translation of the studies described above, have now led to the recent initiation of a clinical trial in which patients with advanced CEA-expressing carcinomas will be receiving rV-CEA/TRICOM as a primary vaccination followed by booster vaccinations with avipox-CEA/TRICOM; CEA-specific immune responses following these vaccinations will then be monitored. Thus, hypothesis-based experimental studies have translated to a progression of science-based and science-evaluated clinical studies.

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■ STRUCTURAL BIOLOGY

Strategies for Supporting Diversity in the Workplace—How Structurally Different Ligands Bind to Protein Kinase C

Pak Y, Enyedy IJ, Varady J, Kung JW, Lorenzo PS, Blumberg PM, and Wang S. Structural basis of binding of high-affinity ligands to protein kinase C: Prediction of the binding modes through a new molecular dynamics method and evaluation by site-directed mutagenesis. *J Med Chem* 44: 1690-701, 2001.

Plants of the family *Euphorbiaceae* have been used for their medicinal properties since their description two millennia ago (Figure 1). These plants first attracted the interest of the cancer research community in the 1940's and 1950's with the identification of croton oil as the archetypical "tumor promoter" in the two-stage mouse skin carcinogenesis protocol. In the late 1960's, the active ingredients in croton oil were identified as esters of the tetracyclic diterpene phorbol. We have come a long way since then.

We now appreciate that the phorbol esters act as ultrapotent analogs of sn-1,2-diacylglycerol, a ubiquitous lipophilic second messenger generated as one arm of the phosphoinositide signaling pathway. Diacylglycerol binds to C1 domains, the zinc finger structures first identified in the regulatory domain of protein kinase C (PKC). The C1 domains function as hydrophobic switches. Occupancy of a hydrophobic cleft in the C1 domain by diacylglycerol completes a hydrophobic surface on one face of the C1 domain, promoting its insertion into the lipid bilayer, both leading to conformational change in the enzyme associated with activation as well as driving translocation from cytosol to membrane.

Such ligands provide tools to probe the regulation of these receptors as well as potential leads for drug development.

The diacylglycerol signaling pathways show great complexity. On the one hand, diacylglycerol-responsive C1 domains have been found in five families of signaling molecules. In addition to the eight responsive PKC isoforms, there are the PKD family of serine/threonine-specific kinases; the chimaerins, inhibitors of p21rac; the munc-13 family of proteins involved in priming of vesicle fusion; and the RasGRP family, activators of Ras and Rap1. Complementing this diversity in receptors is the bewildering array of structural solutions for achieving high-affinity binding to C1 domains. In addition to the phorbol esters, the ingenol esters and the daphnane esters represent other classes of active diterpenes. Indolactam V (ILV) is an example of an indole alkaloid, the bryostatins are macrocyclic lactones, and aplysiatoxin is a polyacetate.

We know that this structural diversity translates into functional diversity. In stark contrast to the tumor-promoting activity of phorbol 12-myristate 13-acetate (PMA)—the primary active ingredient in croton oil—in many biological systems bryostatin 1 behaves as a functional antagonist of PMA, and prostratin (12-deoxyphorbol 13-acetate) displays potent anti-tumor promoting activity. One theme of our research program is to understand in detail the molecular basis for the interactions of ligands with C1 domains and how structural diversity can translate into diversity of outcome. This effort is grounded in close collaboration between computational chemists, synthetic chemists, and

Figure 1. *Croton tiglium* is the source of croton oil, which has long been used as an herbal medicine (Source: Koehler FE. *Medizinal-Pflanzen* [Medicinal Plants]. 1887).

molecular biologists/biochemical pharmacologists both at NCI and at other institutions.

In our research, we used molecular modeling to predict the binding modes of four ligands for C1 domains. The predicted binding of phorbol 12, 13-dibutyrate, ingenol 3-benzoate, thymeleatoxin (a daphnane ester), and ILV were calculated using newly developed methodology in which both the binding site on the C1 domain as well as the ligand were allowed to remain flexible. The predicted interactions were compared with those for phorbol 13-acetate, for which the X-ray structure had been solved previously. We found that the binding of these ligands to the C1 domain was governed by a combination of multiple specific hydrogen bonds and hydrophobic interactions. Although the C1 domain always used two common hydrogen bond donors and two common hydrogen bond acceptors, the hydrogen bond network with each ligand was different. Previous efforts at the analysis of C1 domain-ligand interactions had often assumed that the ligands should possess a common set of hydrogen bond donors and acceptors that could overlap in three-dimensional space. Our findings reveal that this strategy for identification of a ligand pharmacophore is inherently flawed.

To further extend our understanding of the C1 domain-ligand interactions, we used site-directed mutagenesis to directly assess the contributions of conserved residues around the binding site to ligand binding. Molecular dynamics simulations were used to explore the effect of these mutations on binding-site conformation for comparison with the experimental data on binding affinities. Specific mutations caused either global predicted change in the protein conformation, local change in the binding loop, or little conformational effect. The overall effect of the mutations on binding was a combination of the alterations in the binding-site conformation and in direct contacts.

Our emerging insights into ligand interactions at the diacylglycerol receptors afford the basis for the design of synthetically more accessible ligands than the phorbol esters and indole alkaloids. Such ligands provide tools to probe the regulation of these receptors as well as potential leads for drug development to complement the several PKC-targeted drugs already in clinical trials.

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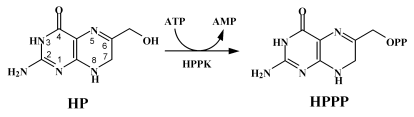
Structure-based Drug Design Is Alive and Well!

Blaszczyk J, Shi G, Yan H, and Ji, X. Catalytic center assembly of HPPK as revealed by the crystal structure of a ternary complex at 1.25 Å resolution. *Structure Fold Des* 8: 1049-58, 2000.

Shi G, Blaszczyk J, Ji X, and Yan H. Bisubstrate analog inhibitors of 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase: Synthesis, biochemical and crystallographic studies. *J Med Chem* 44: 1364-71.

There has long been a promise for rational and structure-based drug design, and many investigators and institutions have struggled along this path. The current healthcare crisis of antibiotic resistance provides a critical arena for this type of research. Xinhua Ji, Ph.D., of the Macromolecular Crystallography Laboratory, has spearheaded a multi-disciplinary project to utilize structure and structure-based drug design to discover new, unique antibiotics. His work has focused on the structure, mechanism, and structure-based inhibitor design for the 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK) enzyme.

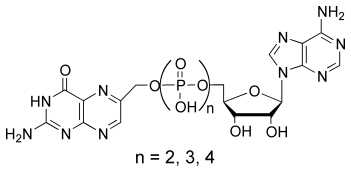
HPPK catalyzes the pyrophosphoryl transfer from ATP to 6-hydroxymethyl-7,8-dihydropterin (HP, see scheme below), the first reaction in the folate biosynthetic pathway.



Folate cofactors are essential for life. Mammals have an active transport system for deriving folates from their diet. In contrast, most microorganisms must synthesize folates *de novo*, because they lack the active transport system. Therefore, HPPK is an ideal target for the development of novel, nontoxic antimicrobial agents, which are urgently needed to combat the worldwide crisis of antibiotic resistance. Because of its small size and high thermal stability, *Escherichia coli* HPPK is also an excellent model enzyme for studying the mechanisms of enzymatic pyrophosphoryl transfer, of which little is known.

In collaboration with Dr. Honggao Yan and coworkers (Department of Biochemistry and Molecular Biology, Michigan State University), Dr. Ji's group has determined the crystal structures of HPPK in its ligand-free form and in complex with HP, two magnesium ions, and AMPCPP (an ATP analog that inhibits the enzymatic reaction), at 1.50 and 1.25 Å, respectively. The comparison of these two crystal structures reveals dramatic conformational changes in three flexible loops and many side chains and thus suggests possible roles of 13 amino acid residues that are strictly conserved among HPPK isoforms found in 11 different species.

Dr. Ji and his colleagues have also determined the crystal structures of HPPK in complex with product molecules, selected inhibitors, and transition-state analogs. Having elucidated this series of high-resolution (up to 0.89 Å) crystal structures of well chosen complexes, they essentially mapped out the trajectory of the HPPK-catalyzed reaction together with the substantial and unusual conformational changes that accompany substrate binding and product release. Of particular importance is the structure of HPPK•HP•$2Mg^{2+}$•AMPCPP, which has been the basis of Dr. Ji's structure-based inhibitor design effort because it mimics most closely the ternary complex of the enzyme and reveals the atomic details of the catalytic assembly. In collaboration with Dr. Yan and coworkers, Dr. Ji's group has carried out the design, synthesis, biochemical, and crystallographic studies of three bisubstrate-mimicking analogs, each of which consists of a pterin, an adenosine moiety, and a linker composed of two to four phosphoryl groups (see scheme below).



In collaboration with Dr. Christopher Michejda and coworkers (Molecular Aspects of Drug Design Section, Structural Biophysics Laboratory), Dr. Ji and coworkers synthesized an HP analog. The crystal structure of HPPK, in complex with this inhibitor and MgAMPCPP, was determined. Further modification and improvement of both types of inhibitors are currently being undertaken based on the structural and kinetic information. The combined efforts of these investigators are continuing to develop new, potent, and specific antibiotics. This multidisciplinary program stands as an excellent example of the type of work that will become part of the CCR's Molecular Targets Initiative.

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Why Single-molecule Biology?

Leuba S, and Zlatanova J. Single-molecule biochemistry coming of age. *Prog Biophys Mol Biol* 74: V, 2001.

Zlatanova J, Lindsay SM, and Leuba SH. Single-molecule force spectroscopy in biology using the atomic force microscope. *Prog Biophys Mol Biol* 74: 37-61, 2001.

Bennink ML, Leuba SH, Leno GH, Zlatanova J, de Grooth BG, and Greve J. Unfolding individual nucleosomes by stretching single chromatin fibers with optical tweezers. *Nature Struct Biol* 8: 606-10, 2001.

Until recently, most discoveries in the molecular life sciences were derived from the average measurements of a population of molecules. Recent advances in instrumentation, however, have now made it possible to observe single macromolecules in real time, thus providing researchers with new insight into their dynamic behavior, and a means of exploring heterogeneity among different molecules within a population.

Our laboratory's main interest lies in understanding the structure and function of chromatin, the complex of DNA and small basic proteins, known as histones, that exist in the eukaryotic nucleus. Chromatin not only packages DNA into the small nucleus, but it also actively participates in gene regulation. Little is known about the forces that ensure the integrity of the structure of chromatin; moreover, its structure seems to be modulated by post-synthetic modifications to both the DNA and the histones. Single-molecule approaches will assist investigators in gaining an understanding of how chromatin structure is related to its function.

The above-referenced articles illustrate some of the ways that single-

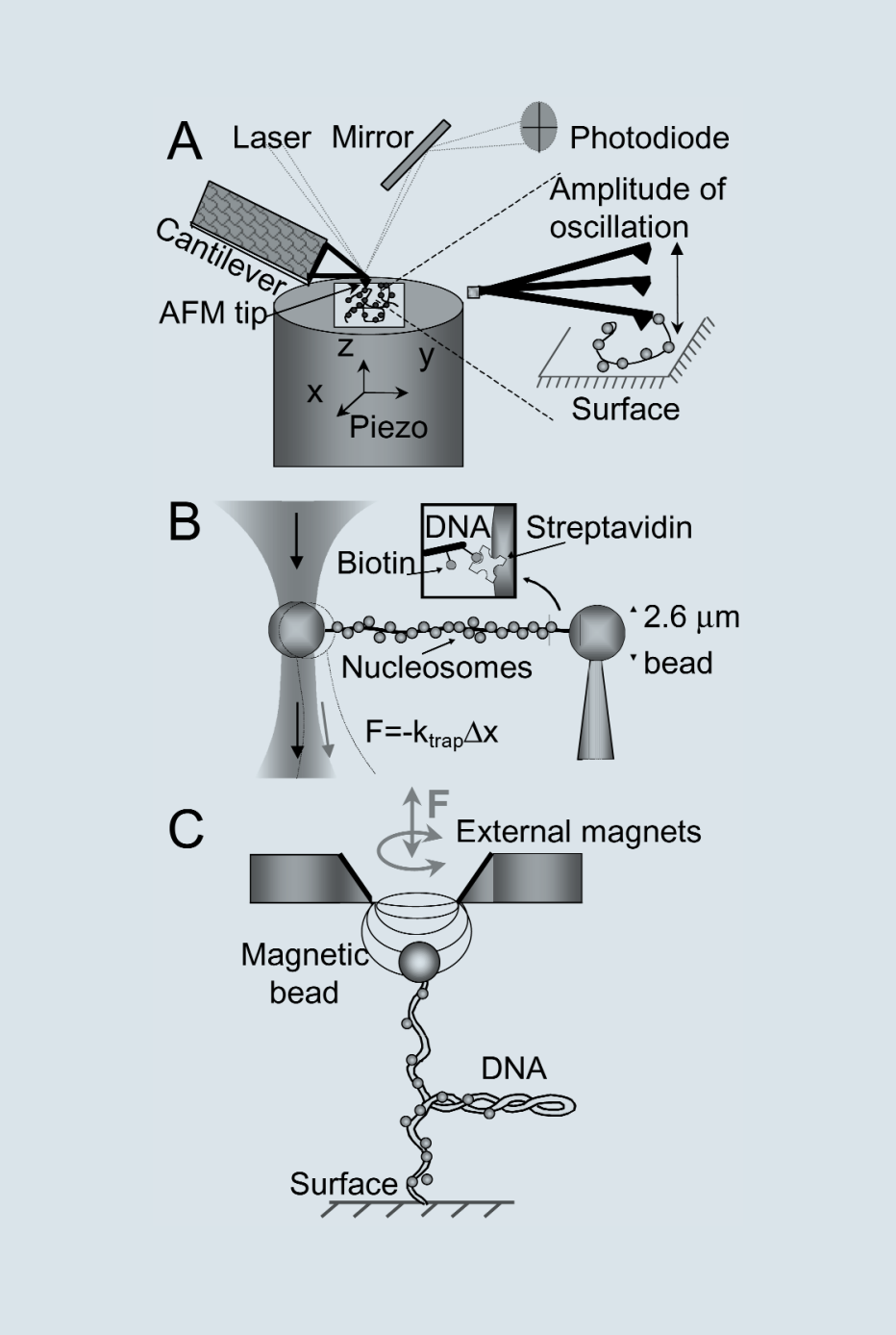

Figure 1. Schematics (not to scale) of the operation of some single-molecule approaches. (A) AFM. A laser is reflected off the backside of the cantilever, bearing a sharp tip. The change in the reflection of the laser at the photodiode determines the topography of the scanned image. A collection of AFM chromatin images can be viewed at http://rex.nci.nih.gov/RESEARCH/basic/lrbge/leuba.html. (B) Chromatin stretching with an optical trap/tweezers. A collection of videos and animations demonstrating real-time attachment of the single λ-DNA to the beads, chromatin assembly, and stretching-induced disassembly can be accessed at http://rex.nci.nih.gov/RESEARCH/basic/lrbge/pmbmov.html. (C) Chromatin stretching with magnetic tweezers.

molecule approaches can be used. The first two articles appeared in a special January issue of *Progress in Biophysics and Molecular Biology,* which presented a series of reviews devoted to "Single-molecule Biochemistry and Molecular Biology" that I co-edited with Dr. Jordanka Zlatanova. A second special issue focusing on single-molecule approaches in biochemistry and molecular biology was published in June 2001 by this same journal. An earlier series of reviews on this subject was edited by Dr. Ken E. van Holde (*J Biol Chem* 274: 14515, 1999).

The Atomic Force Microscope (AFM; Figure 1A) can be used to image protein/DNA complexes and manipulate these complexes in real time. In our review, we focused on the use of AFM to measure interaction forces between or within macromolecules; the measurements are based on Hooke's law ($F = -k\Delta x$, where k is the cantilever spring constant and Δx is the deflection of the cantilever).

Because AFM is a surface-based technique, it may be prone to artifacts. This turned out to be the case when we tried to use AFM to measure the forces holding a nucleosome together (Leuba SH, et al., *Single Mol* 1: 185-92, 2000). As an alternative, we used an optical trap/tweezers (Figure 1B; Bennink ML, et al., *Nature Struct Biol* 8: 606-10, 2001). An optical trap/tweezers can be explained by the following analogy: If the upward airflow from a tube were used to support a plastic globe—similar to an aeronautical exhibit such as the one found at the National Air and Space Museum—and a person pushed the globe slightly out of the vertical flow of air and then released his hand, the globe would fall back into the center of the airstream. To move the globe out of the center of the airflow, the person would need to use a certain amount of force.

After linking a single naked DNA molecule between two polystyrene beads, we flowed in a *Xenopus laevis* egg extract that assembled nucleosomes onto the DNA (to view a video of the assembly process, go to http://rex.nci.nih.gov/RESEARCH/basic/lrbge/pmbmov.html). We then stretched the assembled chromatin fiber by moving one of the beads (held by suction in a pipette) away from the bead that was held in the optical trap while monitoring its deflection. Quantized force disruptions were observed at approximately 65 nm increments; these increments reflect unraveling of individual nucleosomes. Essentially, we have observed the force (approximately 20 to 40 picoNewtons) required to unravel individual nucleosomes. Next, we wanted to determine what would happen if acetylated (or phosphorylated, ubiquitinated, etc.) histones were used to assemble nucleosomes? Would we detect a decrease in the measured force? These are questions that now can be addressed using this approach.

Another new optical detection method employs magnetic tweezers (Figure 1C). In this technique, both ends (all four strands) of the linear DNA are cemented to the microscope glass surface as well as to a magnetic bead to topologically constrain the molecule. Rotating external magnets then introduce positive or negative supercoils in the DNA in a controlled way. It has already been shown that it is possible to measure the twist of the DNA, to determine length reductions resulting from plectoneme formations, and to measure the relaxation of individual plectonemes to topoisomerase II (Strick T, et al., *Prog Biophys Mol Biol* 74: 115-40, 2001). We plan to use this approach to study how torsion and transcription affect individual nucleosomes.

The use of these novel single-molecule approaches opens up an entire new field of investigation and holds the promise of answering questions that have not been easily addressed using population-average methods. Researchers are using these techniques to determine the forces that different classes of proteins (enzymatic machineries such as polymerases and helicases or architectural proteins such as histones and high-mobility group proteins) apply to the DNA while performing their functions.

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Administrative Links

Cancer Mortality Maps and Graphs Web site

NCI has released the new Cancer Mortality Maps and Graphs Web site, which you can access at http://www.nci.nih.gov/atlasplus/.

Comments, suggestions, or questions can be submitted by email to Division of Cancer Epidemiology and Genetics directly from the site by clicking the "Contact Us" link.

Name Change

The NCI Technology Development and Commercialization Branch (TDCB) has changed its name to the NCI Technology Transfer Branch (TTB). Visit the TTB Web site at http://ttb.nci.nih.gov/.

NCI Faculties Listserve Sign-up

You are invited to sign up for any of the NCI Faculties by going to the following site: http://dcs.nci.nih.gov/faculties/facultylistserves.cfm.

You can also access this site from the NCI CAMP site at http://camp.nci.nih.gov/Default.htm by clicking on **Intramural Science**. The link can be found under the **Center for Cancer Research (CCR)** heading within the main heading **Organizations.**

Student Loan Repayment

The Office of Personnel Management (OPM) has proposed final regulations to implement provisions authorizing federal agencies to repay federally insured student loans, when necessary, in order to recruit and retain highly qualified personnel. Richie Taffet, from the Human Resources Management and Consulting Branch, represents NCI on the NIH Working Group, which is engaged in establishing a loan repayment program at NIH. For the complete story, click on http://camp.nci.nih.gov/admin/news/admin/200107/loan.htm.

Classification Change: "Information Technology Management Series"

The OPM has created a new occupational group, the "Information Technology Management Series," to give federal agencies more flexibility in assigning salaries and promoting high-tech personnel. Most of the high-tech individuals who were previously designated as computer specialists will be moved into this new series as information technology specialists. For the complete story, click on http://camp.nci.nih.gov/admin/news/admin/200107/classification.htm.

Conflicts of Interest

The NCI Ethics Office addresses the following areas of conflict and how to deal with them: actual conflict of interest, appearance of a conflict of interest, cautionary note, disqualification (recusal), authorization, regulatory exemption, waiver, resignation from an outside position, divestiture, and reassignment. For more information, click on http://camp.nci.nih.gov/admin/news/admin/200107/remedies.htm.

Federal Employees and Foreign Governments

A federal government employee ***may not*** accept employment or a title from any foreign government. For more information, visit http://camp.nci.nih.gov/admin/news/admin/200112/ethics.htm.

Federal Employees and Outside Activities

In addition, when performing a personal outside activity such as teaching, speaking, editing, or writing, you **must always identify** that you are performing this activity in your own personal capacity—it must be clear that you are not representing the NIH. For more information, visit http://camp.nci.nih.gov/admin/news/admin/200110/title.htm.

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