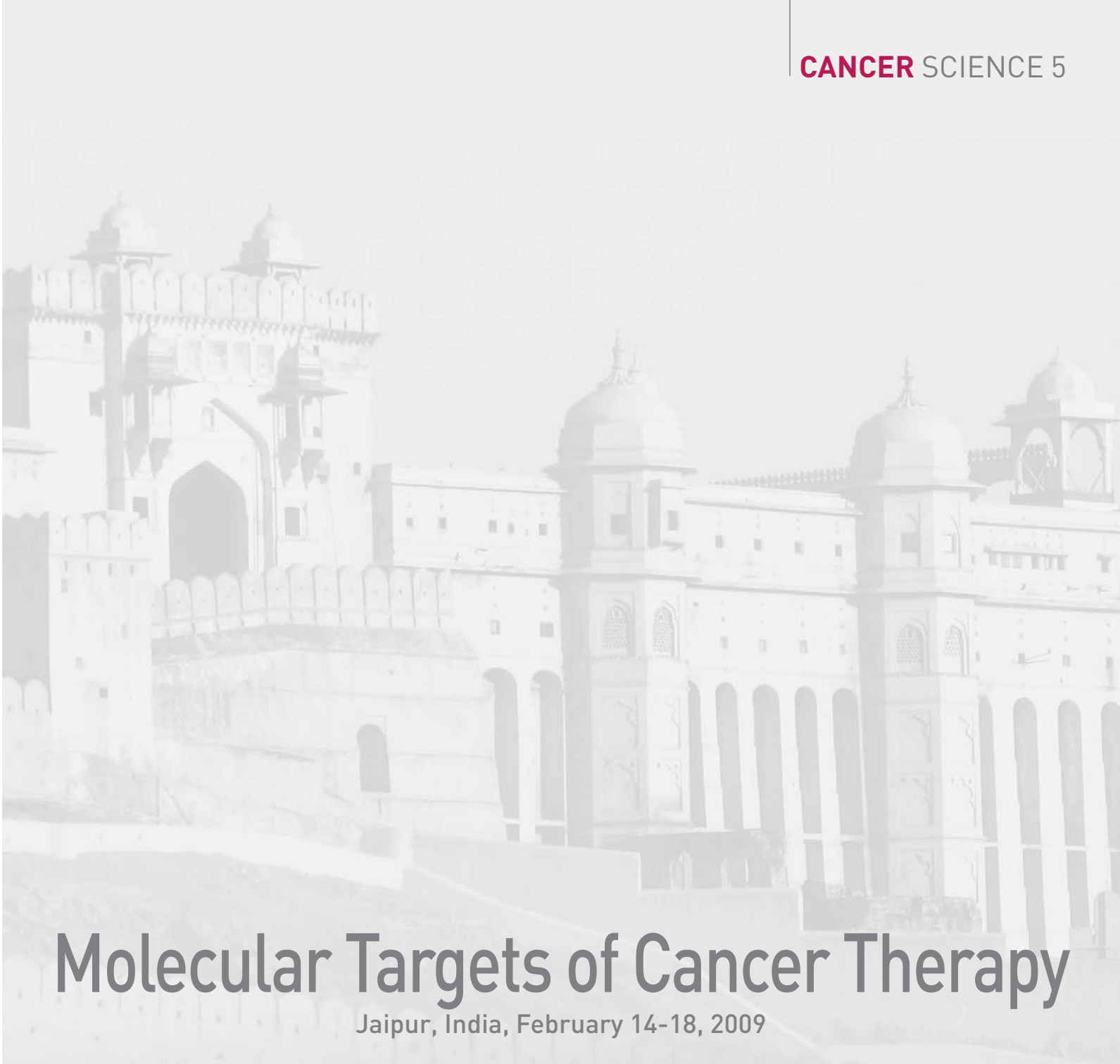

Molecular Targets of Cancer Therapy

Jaipur, India, February 14-18, 2009

COLLOQUES MÉDECINE ET RECHERCHE

FONDATION IPSEN

SCIENTIFIC REPORT BY APOORVA MANDAVILLI

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FOREWORD BY INDER M. VERMA

Exactly five years ago, the first of Fondation Ipsen's cancer series meeting was held in Agra, India, the home of the Taj Mahal. The subject was, **Can Cancer be treated as a chronic disease?** The exceptional success of Gleevec, inhibitor of the BCR-ABL kinase, made it the poster child for success in cancer treatment. Gleevec's use in other cancers, such as gastrointestinal stromal tumors, was just being explored.

Nearly all patients with chronic myelogenous leukemia have the same molecular abnormality that leads to constitutive activation of the BCR-ABL kinase, which activates signals leading to enhanced cell survival and unchecked proliferation. However, in other cancers, only a subset of patients have a mutation in their tumor cells that alters the biological potency of the target kinase and makes the cell sensitive to kinase inhibitors. This was exemplified by the epidermal growth factor receptor (EGFR) kinase inhibitors, which showed dramatic regression of tumors in only a subset of patients with non-small cell lung carcinoma.

During February 14-18 of 2009, we revisited the subject of **Molecular targets of cancer therapy** in Jaipur, India, the princely city renowned for its unique architecture and fabulous palaces. **Harold Varmus** opened the meeting with a review of the progress in therapeutic strategies with special emphasis on lung and breast cancer. In particular, he described approaches to developing targeted therapies for EGFR-mutant lung cancer.

John Kuriyan also focused on EGFR, describing the molecular and biophysical mechanisms underlying the receptor's activation, knowledge of which could lead to rational drug design. The HER family of receptors have also proven to be successful targets in cancer therapy. **José Baselga** described clinical trials under way with various HER2, HSP90, PI3K and IGF1R inhibitors to treat breast cancer.

Several talks emphasized the potential for targeting oncoproteins as a treatment for cancer. **Hugues de Thé** suggested that targeting oncoproteins for degradation could serve as a general strategy for fighting cancers in which the acquisition of stemness results from oncogene expression. **Julian Adams** presented details of inhibitors of the heat shock protein HSP-90, elevated levels of which are associated with various cancers. **Michael Bishop** explained that, in at least some instances, treatment outcomes for cancer can be improved by directing two distinctive therapeutics at the same molecular target.

Clinical strategies to mobilize the immune system against cancer cells have so far been disappointing. **James Allison** suggested that combinations of conventional therapies with immune checkpoint blockade may effectively treat cancers and minimize the chances of tumor resistance and escape. Another strategy, suggested by **Alexander Levitzki**, is to use a ligand-guided vector carrying a double-stranded RNA that would trigger the production of cytokines and chemokines, which in turn attract immune cells to the tumor.

The nature of immune reactions that may control tumor invasion and metastasis is also of seminal importance for identifying novel targets for cancer treatment. **Wolf-Hervé Fridman** presented data showing that agonists for the Toll-like receptors TLR7 and TLR8 up regulate BCL-2 expression, increase

tumor cell survival and promote chemoresistance — factors to be taken into consideration before administering TLR agonists as vaccine adjuvants to treat cancer.

The comprehensive annotation of human cancers at the molecular level is becoming ever more a reality, enabled by a number of large-scale public efforts. **William Sellers** described examples in which information about cell lines at a genetic or molecular level has helped make robust predictions of clinical response. In particular, Sellers presented data on BEZ235, a dual mTOR/PI3K inhibitor in phase 1 clinical development. **Kevan Shokat** reported on another mTOR inhibitor, PP242, whose enhanced ability to block mTOR signaling is based on its more effective mTORC1 inhibition than that of rapamycin.

Apart from the PI3K signaling pathway, aberrant activation of the Hedgehog pathway, an ancient signaling cascade that is crucial for proper development, can lead to cancer. **Frederic de Sauvage** explained that targeting the Hedgehog pathway with small molecule antagonists provides a new therapeutic opportunity for the treatment of those tumors. Inhibitors of histone deacetylase (HDAC) represent another promising new class of anti-tumor agents. **Victoria Richon** presented data on vorinostat, the first HDAC inhibitor approved for the treatment of cancer.

The speakers also discussed other strategies for drug discovery in cancer. **Arul Chinnaiyan** described one of the first unbiased assessments of metabolites in cancer development. As an alternative to direct targeting of oncogenes, **Gary Gilliland** elaborated on 'synthetic lethality' screens for genes that are essential only in the context of specific cancer-causing mutations. Finally, **Gregory Verdine** presented progress on the development of synthetic biologics for so-called undruggable targets. As chair of the last session, **Tak Mak** highlighted the rationale and excitement of targeting specific biochemical pathways involved in metabolism.

Overall, there was great optimism despite the many problems encountered by the newer therapeutic agents in the clinic. It seems that the systematic study of the cancer cell in the past 40 years — when President Richard Nixon declared war on cancer — are beginning to pay off.

It is clear that there will not be any one "wonder drug" that can cure all cancers. But it is also quite encouraging to note that a combination of drugs attacking different pathways can control the unchecked spread of many otherwise lethal cancers.

As in the past four meetings, the wonderful ambience, the beautiful palace grounds, and the immaculate care and organization by Jacqueline Mervaillie , Yves Christen and their staff made this meeting very special and enjoyable. Finally a very special thanks to Apoorva Mandavilli, who even at her advanced stage of pregnancy took diligent notes, which has led to this very comprehensive and readable monograph.

Inder Verma

PART I: Targeting receptors

Targeted therapies

A report on a lecture by
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Lung cancer is the leading cause of death from cancer. Therapies targeted against specific genes and proteins have offered some promise in treating lung cancer, particularly adenocarcinoma, the most common form of the disease. Mutations in the epidermal growth factor receptor (EGFR), which are responsible for about 10 percent of lung adenocarcinomas, render the EGFR kinase hypersensitive to tyrosine kinase inhibitors such as gefitinib (Iressa) and erlotinib (Tarceva). However, in the long term, most patients go on to develop resistance to these therapies and succumb to the disease. Relying on human tissue samples, mouse models and cancer cell lines, researchers are trying to achieve a deeper understanding of the molecular mechanisms underlying adenocarcinomas. They are also using a number of structural and functional approaches to overcome resistance by identifying specific mutations that confer resistance, and screening for new drug candidates. ***Harold Varmus*** *described approaches to developing targeted therapies for EGFR-mutant lung cancer.*

Targeted therapy, although it is not yet in widespread use in oncology, has enjoyed a few signal successes, most dramatically in the use of imatinib (Gleevec), a tyrosine kinase inhibitor, to treat chronic myeloid leukemia. What's more, most cases of resistance to imatinib — caused by a series of mutations affecting BCR-ABL — can be treated with two new drugs, dasatinib and nilotinib.

Although successful targeted therapies have not yet been developed for most cancers, there are some intermediate successes — for example, the tyrosine kinase inhibitors erlotonib (Tarceva) and gefitinib (Iressa), both of which induce at least a partial response in most EGFR-mutant lung adenocarcinomas.

However, within a year or two, the disease progresses as a result of drug resistance, attributable in about half of the cases to secondary mutations in EGFR and in some others to amplification of the MET gene. These findings have focused attention on the need to find drugs that overcome resistance and to identify other resistance-inducing changes.

There are a number of limitations that can explain why responses to targeted therapies do not occur, are incomplete, and are not well sustained. Researchers have an incomplete view of the genotype in nearly all cancers, so it is not known what other genes are mutated besides EGFR, and what consequences those mutations might have for cell signaling and successful drug development.

Developing better drugs against known targets, against additional targets in the same or other signaling pathways or against new targets may all help solve these challenges. But researchers are constrained by the kinds of available materials and experimental approaches.

For most of these diseases, there are samples of human tumors either stored in freezers, or newly available for full structural characterization. These tumors can be analyzed for mutations and changes in gene expression, protein modification and chromatin structure. Many of

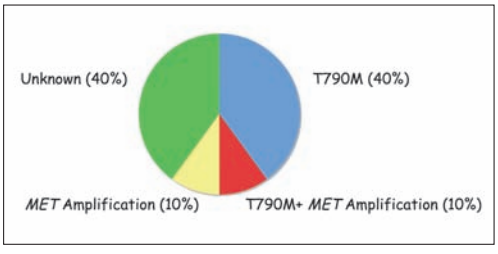

Figure 1
The mechanism of acquired resistance to tyrosine kinase inhibitors is unknown in approximately 40% of cases.

these analyses are being undertaken in the international effort to build The Cancer Genome Atlas.

Mouse models are also useful in this quest, especially when the mouse cancers are generated by mutations also encountered in the analogous human tumor, and when they show similar pathophysiology. These models can help assess drug resistance, tumor progression, and tumor recurrence after remission. They can also be used in pre-clinical tests of therapeutic strategies, and help assess the roles of various signaling pathways.

A third approach is to use cancer cell lines, or in some cases, transformed cells of the appropriate tissue type. These allow for a deeper study into the nature of the target and its interaction with drugs, mainly using biochemistry on proteins and signaling pathways, screening for new targets with drugs or inhibitory RNAs and validating those targets.

EGFR mutations

Lung cancer is the most common cause of death from cancer, killing roughly a million people worldwide each year. Based on histopathology, there are four major types of lung cancer: small cell lung cancer, large cell carcinoma, adenocarcinoma, and squamous cell carcinoma; adenocarcinoma is the most common of these.

EGFR-mutant tumors account for about 10% of all adenocarcinomas, and two mutations dominate. Roughly 45% are deletions of a sequence that encodes the "LREA" motif that lies very close to the ATP binding site at position 745. Another 40% to 50% are point mutations that change a leucine to arginine at position 858. There are also a few other less common mutations that correlate with clinical responsiveness to tyrosine kinase inhibitors (see Kuriyan, page 13).

About half of all tumors that acquire resistance to tyrosine kinase inhibitors have a T790M mutation in the kinase domain of EGFR[1]. This is highly analogous to an imatinib-resistance conferring mutation in BCR-ABL, T315I, the so-called gatekeeper mutation that affects ATP binding.

Although there are some other mutations that appear to confer resistance to inhibitors, there is no explanation for resistance in about 40% of patients. Some groups have observed MET amplification with or without concurrent T790M mutations, but the remainder are unexplained.

In the past few months, several institutions have teamed up on a sequencing project to evaluate as completely as possible the genetic lesions in adenocarcinomas of the lung. The more recent of the two papers published by the consortium[2] analyzed 188 adenocarcinomas for the sequence of the coding exons of 623 genes.

The researchers confirmed the high mutation rate in a number of genes known to be mutated such as p53, KRAS and EGFR, but also revealed a number of unexpected genes to be mutated, including NF1, ATM, APC, EPHA3 and various others. The findings offer new potential targets for therapy in those selected patients who have these mutations. This large dataset also reveals combinations of mutations that can inform the development of better targeted therapies.

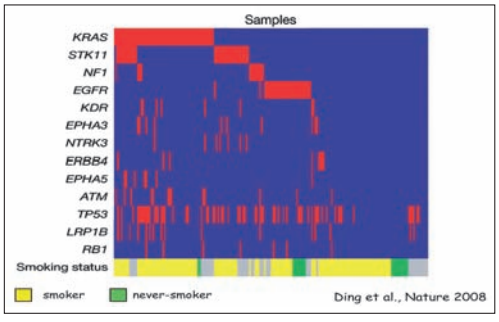

Figure 2
Some mutations are mutually exclusive, some are not.

For example, it's been known for some time that mutations in KRAS, which account for 25% of the tumors, don't occur in concert with mutations in EGFR, presumably because both are members of the same signaling pathway. But mutations in NF1, which regulates the activity of RAS and RAS-like proteins, can co-exist with mutations in KRAS.

Interestingly, a number of other transmembrane tyrosine kinases are mutated, including members of the TRK family, Ephrins and ERBB. When mutations occur

in any of the other receptors that have kinase activity, EGFR is not mutated. Also, perhaps not surprisingly, mutations in ATM do not co-exist with mutations in p53.

Another advantage of this sort of analysis is the opportunity to look at tumors in relation to clinical features, such as smoking history. Among non-smokers with lung adenocarcinoma, for instance, EGFR mutations are frequent, affecting roughly 50% of non-smokers with the disease[3]. Still, there's a large block of patients who were never smokers, and do not have mutations in any of the oncogenes and tumor suppressor genes examined thus far.

Mechanisms of resistance

The Varmus laboratory has built mouse models of lung cancer in which a transgene contains either of the two common EGFR mutants, and is expressed in the lung under the control of doxycyline. William Pao at MSKCC has also built similar mouse models in which the mutant alleles in the transgenes contain the T790M resistance-conferring mutation.

In these models, doxycycline induces adencarcinoma within a few weeks or months, and the tumors regress rapidly when the doxycycline is removed. Unless the T790M mutation is present in the transgene, persistent erlonotib treatment results in complete remission.

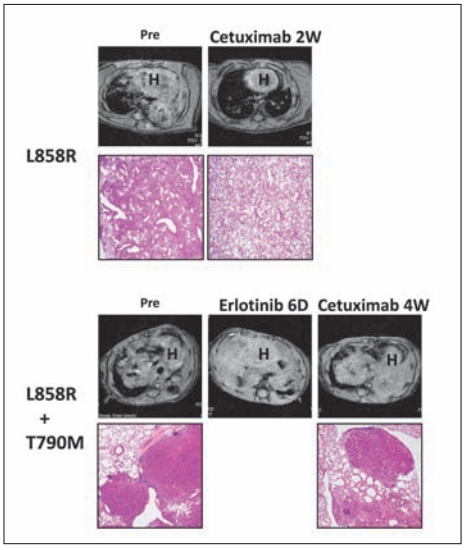

Figure 3
L858R-induced tumors regress with cetuximab, but erlotinib-resistant tumors do not.

Intermittent therapy with erlonotib can be used, however, to recapitulate drug resistance in these animals. Based on magnetic resonance imaging of the animals, tumors decrease dramatically in size when the mice receive erlonotib, but recur when the animals are given a break from the therapy. Ultimately, tumors begin to grow despite the administration of erlonotib.

About 20% of these drug-resistant tumors have acquired the T790M mutation; a few animals have low levels of amplification of the MET proto-oncogene and one has a high level, suggesting that the mouse model recapitulates the same kinds of resistance mechanisms seen in human lung tumors. To identify additional mechanisms of resistance, researchers are undertaking wide-scale comparisons of both genome integrity and gene expression in drug-resistant and untreated tumors.

Other approaches

The four members of the immediate EGFR family of proteins have the ability to form heterodimers, and heterodimerization of EGFR with ERBB2 or ERBB3 might be important in tumorigenesis.

To address whether ERBB3 is required for tumor induction, mice with floxed alleles of ERBB3 were used to show that tumors appear with more or less the same kinetics regardless of the number of ERBB3 genes (0, 1 or 2), implying that EGFR-ERBB3 heterodimers do not need to form to induce tumors with mutant EGFR. Experiments are under way to test whether ERBB3-deficient tumors respond to erlotonib, and whether signaling through PI3K and AKT is impaired.

A high-throughput screening facility at MSKCC has made it possible to screen a few hundred thousand small molecules against a small collection of human adenocarcinoma cell lines with KRAS or EGFR mutations.

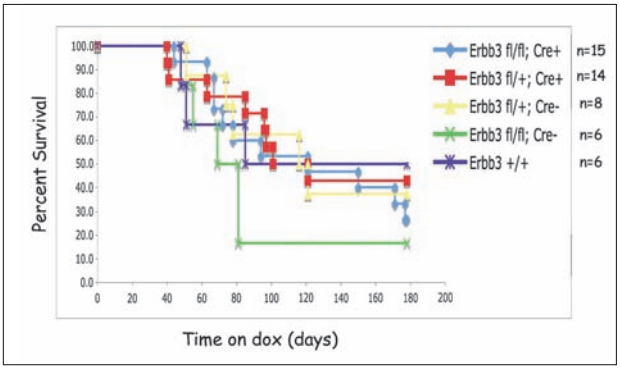

Figure 4
TetO-EGFR-L858R mice develop lung tumors at similar rates with and without ERBB3.

After a number of winnowing steps, the screen has identified four lead compounds with sub-micromolar inhibitory effects on the different cell lines. However, the pattern of inhibition does not correlate with the known mutational status, suggesting that the targets of these compounds are unlikely to be KRAS or EGFR.

For one of these compounds, 43 analogs have been synthesized, and structure-activity studies have allowed the production of affinity columns in efforts to define the molecular target. Comparative expression profiling and inhibitory RNA experiments may be useful means to validate any promising targets.

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Activation of the EGF receptor

A report on a lecture by
John Kuriyan
University of California at Berkeley, Berkeley, USA

The epidermal growth factor receptor (EGFR) and its homologs (HER2, HER3 and HER4) comprise an important and distinct subfamily of receptor tyrosine kinases. Upon binding ligand, the extracellular domains of EGFR family members dimerize such that their C-terminal ends are brought close together. One feature that distinguishes the EGFR family from more typical receptor tyrosine kinases is that activation involves the formation of an asymmetric dimer between the kinase domains of two receptor molecules, in which one kinase domain activates a second kinase domain. The juxtamembrane segment of EGFR greatly potentiates the catalytic activity of the kinase core and is distinct from that of other receptor tyrosine kinases. ***John Kuriyan*** *discussed how the juxtamembrane segment latches the two kinase domains together, resulting in their activation. There is also an inactive kinase domain dimer in which the C-terminal tails prevent formation of the juxtamembrane latch. The formation of pre-formed EGFR dimers is likely to constitute a key auto-inhibitory mechanism that prevents ligand-independent activation.*

EGFR is unusual in terms of its molecular mechanism. The textbook model for how a receptor kinase activates is that it involves the formation of a dimer, in which the cytoplasmic kinase domains of the receptor phosphorylate each other on a loop near the active site, in a way that switches on enzymatic activity.

The kinase domains are constructed so that one kinase molecule cannot phosphorylate itself on the activation loop. In the simplest model, a ligand brings two receptor molecules close together or alters their orientation in some way so that the kinase domain of one receptor can phosphorylate and activate the other.

This is how the insulin receptor works, for example. But this model does not apply to EGFR. In a structure determined at Genentech of the complex of the kinase domain of EGFR with the cancer drug erlonitib, the EGFR kinase domain is not phosphorylated, but is still in an active conformation.

This fits with the knowledge that even if a tyrosine residue that would normally be phosphorylated is mutated, EGFR activity is unaffected. The key difference with the canonical model is therefore that EGFR doesn't require phosphorylation on the activation loop for activity.

But this poses a puzzle. EGFR has a long tail, and phosphorylation of tyrosines on this tail creates SH2 docking sites that then transduce the signal. The tail is so long and unstructured in its elements that if the kinase were intrinsically active, as suggested by the Genentech structure, the kinase would phosphorylate itself. So, there must be an auto-inhibitory mechanism that prevents this from happening.

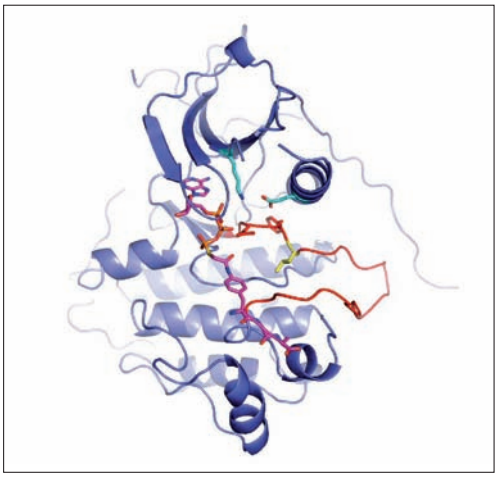

Figure 1
Crystal structures of the EGFR kinase domain reveal an active conformation.

Inactive conformation

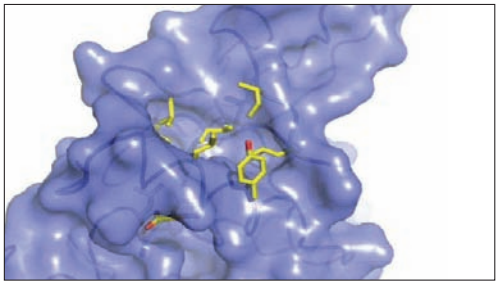

Figure 2
Transition from inactive to active in the EGF receptor.

A structure of the EGFR kinase domain complexed with another cancer drug, lapatinib, broke open the puzzle of how EGFR is kept inactivated before it is switched on[1]. In this structure, which was determined at GlaxoSmithKline, lapatinib is bound not to an active conformation of the kinase domain, but instead to an inactive one. In fact, it's in a specific inactive conformation that has been seen previously in the Src kinases and in the cyclin-dependent kinases. Thus, the EGFR kinase domain is intrinsically auto-inhibited.

A number of studies have noted that a leucine residue (Leu834) is mutated to arginine in several lung cancer cases with EGFR mutations. This finding is consistent with the Glaxo structure of the lapatinib complex, in which this leucine residue stabilizes the inactive conformation.

Mutation to arginine is expected to activate the kinase by destabilizing the inactive conformation. When the EGFR kinase domain is isolated from the rest of the protein, it has very low activity. Making the single leucine 834 mutation to arginine increases catalytic activity substantially[2]. This is an exciting finding because it shows that the kinase domain must indeed be switched off in the absence of EGF, and that lapatinib has trapped the inactive conformation.

Why is the active conformation seen in the Genentech structure of the erlotinib complex? When the kinase domain is crystallized, it is at a concentration in the millimolar range, which is close to the concentration at the membrane when the EGFR molecules are next to each other. Growing the crystal may shepherd the kinase domains together, mimicking what EGFR does when it switches on.

In contrast to lapatinib, erlotinib does not require the kinase domain to be in an inactive conformation, which allows the asymmetric dimer to form in the crystal. If that's the case, the crystal may hold the answer to how the kinase domain is switched on.

Indeed there's an asymmetric interaction between two molecules in the crystal structure of EGFR kinase domains that is reminiscent of the action of a cyclin on a cyclin-dependent kinase (CDK). In the EGFR, an alpha helix has to rotate in order for the kinase to switch on. This rotation is brought about exactly as a cyclin activates a CDK, except that the activator is another molecule of EGFR itself.

These observations suggest that asymmetric interaction between kinase domains underlies the activation. If a single mutation from valine to arginine, V924R, is introduced in the kinase domain at the cyclin-like interface, the prediction would be that the kinase cannot sustain the asymmetric interaction, and therefore cannot activate.

A crystal structure of the kinase domain with this single point mutation reveals it to be in an inactive conformation, one that resembles closely the structure of the lapatinib complex. Lapatinib doesn't induce any structural changes, it just recognizes the natural inactive conformation of the EGFR kinase domain.

Latching domains

How does EGF activate the EGF receptor? Clues to the mechanism come from studies on a portion of the receptor that's just next to the membrane on the cytoplasmic side, called the juxtamembrane (JM) segment. The first clue to this domain's role came from a paper last year, which showed that the intracellular portion of EGFR alone — without the extracellular or transmembrane domains, but including the JM segment — can activate the kinase domain by stabilizing the asymmetric dimer[3].

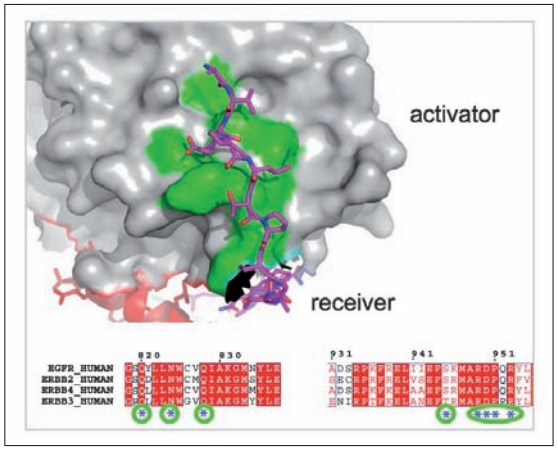

Figure 3
The juxtamembrane latch.

The JM-segment has two portions, an N-terminal JM-A segment and a C-terminal JM-B segment. Another clue to the mechanism came from analyzing a crystal structure of the HER4 kinase domain, again from Glaxo, bound to an inhibitor that formed a covalent bond to the kinase domain. It turns out that in this crystal structure the JM-B segment from the receiver kinase domain wraps around the activator kinase domain, forming a latch-like structure, and hooking the kinase domains together[4].

Intriguingly, both the surface of the activator — the part that the JM segment interacts with — and the JM segment itself are highly conserved among EGFR family members. Mutating either the individual residues in the JM latch, or the residues that interact with them, seriously affects the ability of the kinase to activate.

Deleting the JM segment on either the activator or the receiver also kills the activity. These observations suggest that the JM segments on both kinases must be interacting in some way.

This idea is further verified by experiments that measure the concentration dependence of activity. If the EGFR kinase domain dimerizes through the JM segment, it must be concentration dependent. Sure enough, increasing the concentration of the wild type kinase domain and the JM domain to the micromolar range results in activation.

If the JM-A segment alone is deleted, the dimerization becomes much weaker; the JM-A segment is required on both the activator and the receiver kinase domain.

These results lead to the conclusion that the JM-A segment is part of the structural latch that holds two kinase domains together in the activated asymmetric dimer.

The sequence of the JM-A segment suggests that it is helical, with more negative residues on one end and more positive resides on the other. This led to the idea that the interaction between two JM-A segments involves the formation of an antiparallel dimeric coiled-coil.

Crossing helices

Last year, a research team in Russia used NMR analysis on the HER2 transmembrane domain to determine its structure in a lipid bilayer. The published structure is consistent with previous predictions for the transmembrane segment, with the two helices crossing like chopsticks. The splayed apart transmembrane helices can be readily docked onto the antiparallel helices of the juxtamembrane segment.

One conclusion from this work is that the juxtamembrane segment synergizes with the transmembrane segments in a way that switches on the kinase domains without any necessity for the extracellular domains. The primary function of the extracellular domains may be to prevent the transmembrane and cytoplasmic portions of the receptor from getting together in a productive way.

Combining these ideas with previously available structures for the extracellular portions of EGFR allows a model for the entire activated EGFR to be generated.

In this model, the JM segment first latches two kinase domains together to form an asymmetric dimer, and further stabilizes the dimer by forming an anti-parallel helix, which couples to the dimeric transmembrane

helices. The crystal structure of the extracellular domain has shown that binding of EGF brings the tips of the extracellular domain together. That docks on to the transmembrane domain, which then docks on to the anti-parallel helix formed by the juxtamembrane segment.

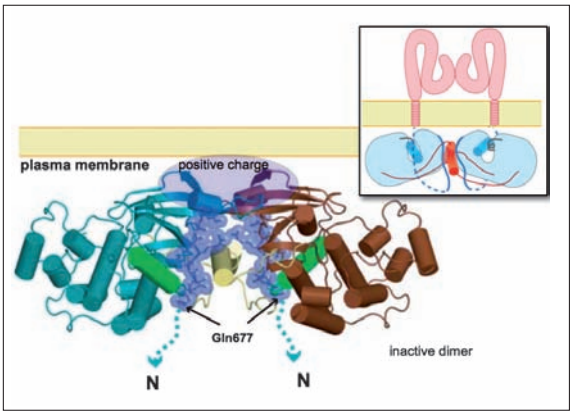

Figure 4
A model for the generation of active EGFR.

The potent ability of the juxtamembrane and transmembrane segments to activate EGFR suggests that there will be inhibitory mechanisms that prevent this from happening in the absence of EGF, in addition to that provided by the extracellular domains.

Crystallography has revealed an inactive dimeric form of the EGFR kinase domain, in which the C-terminal tails are arranged such that they block the formation of the juxtamembrane latch[8]. The first portion of the C-terminal tail encompasses a helix, which is the docking site for the clathrin adaptor AP-2, for a component of one of the pathways by which EGFR is internalized. This helix is completely buried in the inactive dimer, and hides the endocytosis signal.

The helix also guides the subsequent portion of the C-terminal tail such that it runs along the surface that is used to engage the JM-B segment when the activating latch is formed. The crystal structure also suggests that positive charges on one side of the inactive dimer position it at the membrane in a way that prevents the formation of the anti-parallel coiled coil by the JM-A segments.

In the absence of ligand, there is likely to be an equilibrium between the inactive monomeric and dimeric forms of the receptor. EGF shifts the balance by promoting the interaction between the helices and the anti-parallel helix, switching the population to the active dimeric form.

One important avenue to explore in the future is the development of small molecules that prevent the cytoplasmic domains from forming the asymmetric and active dimer. Such molecules would be an important addition to the arsenal of EGFR blockers that are currently in the clinic.

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Emerging molecular targets in breast cancer: HER2 and beyond

A report on a lecture by
José Baselga
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Despite the recent advances in the therapy of HER2-positive breast cancer, primary and acquired resistance to trastuzumab remains a formidable challenge. Improved insights into the biology of the HER family of receptors have led to novel and active anti-HER2 therapies including tyrosine kinase inhibitors, antibody-based conjugates and HSP-90 inhibitors. Combined anti-HER2 therapies may be superior to single agent strategies. For example, the combination of trastuzumab and lapatinib is superior to either agent given alone. Emerging appreciation of the critical role of HER2:HER3 heterodimers has also led to the clinical development of antibodies such as pertuzumab that act as dimerization inhibitors. Mechanisms of resistance to anti-HER2 agents include the presence of a truncated form of the receptor, activation of the insulin-like growth factor receptor and deregulation of the PI3K pathway. Tumors expressing the truncated receptor may be sensitive to tyrosine kinase inhibitors or to HSP-90 inhibitors. In the case of aberrant activation of the PI3K pathway, there is a synergistic functionality between trastuzumab and PI3K inhibitors in HER2-positive cells harboring mutations in the PI3K pathway. ***Jose Baselga*** *described clinical trials under way with various HER2, Hsp90, PI3K, and IGF1R inhibitors to treat breast cancer.*

The clinical landscape for breast cancer looks promising, with several new potential targets having been identified, and molecular agents that interfere with their function being developed. The list of candidates includes new molecular targeted agents directed at the HER family, PI3K, AKT, mTOR and others and angiogenesis inhibitors such as bevacizumab (Avastin) and low molecular weight tyrosine kinase inhibitors.

For many years, breast cancers were classified as either estrogen receptor(ER)-positive (also known as Hormone Receptor (HR) positive) or ER-negative. Today, there are at least three well-defined clinical subtypes: HR-positive , HER2-amplified, and HR-and HER2-negative, also known as triple negative or basal like. Even within these three major subtypes, further subgroups have been defined. For example, within the HR-positive group, two subsets, the luminal A and luminal B behave differently. The same applies to the basal type cancers, and to the HER2-positive subgroups.

HER2 is a tyrosine kinase cell surface receptor that is a member of the human epidermal growth factor receptor family, or HER, a family of receptors that is frequently dysfunctional in cancer. There are four members of this receptor family, although HER2 is clearly the most relevant in breast cancer. HER2 has no known ligand, but has the most signaling capacity. HER3 has no signaling capacity, and yet is critically important because an array of ligands bind to it and trigger activation of signal transduction pathways.

HER2 is amplified in up to an estimated 20% of breast cancers. Clearly, aberrant HER2 expression and functionality results in a more aggressive phenotype. Only 15 years ago, having a HER2-positive tumor was considered very bad news. Survival in patients with HER2 tumors was half of that of those who didn't have HER2 amplification.

Trials with the monoclonal antibody trastuzumab (Herceptin) were the beginning of the change in the history of this disease. It is now well established that HER2 is a potent target for anticancer therapies, and there are a plethora of available agents, including antibody-based therapies as well as small tyrosine kinase inhibitors.

In the case of HER2, available agents such as trastuzumab have markedly increased the cure rate of patients with early-stage breast cancer. In patients with metastatic disease, however, the majority of tumors eventually recur and result in the patients' death. New agents are needed, as is an understanding of the mechanisms of primary and acquired resistance, in order to individualize therapy and design rationally-based combination therapies.

Among patients with early-stage breast cancer, more than 85% of those given trastuzumab during one year are disease-free, without recurrence, after two years.

Novel therapies

Since those initial studies with trastuzumab, several new ways have emerged to target the HER2 receptor. Trastuzumab binds to domain IV in the receptor's extracellular domain, which is very close to the transmembrane region. Other monoclonal antibodies bind to other domains of the extracellular domain of the receptor and also have a potential therapeutic benefit.

For example, pertuzumab is the humanized equivalent of the murine 2C4 and binds to the dimerization domain, interfering with dimerization of HER2. Then, there is trastuzumab-DM1, an antibody-toxin conjugate that consists of a trastuzumab molecule linked to DM1, a vinca alkaloid that is 10,000 times more potent than vinorelbine. This complex is internalized, and DM1 is released inside cells that have HER2 over-expression.

There are new bi-specific and tri-specific antibodies that bind not only to HER2, but also to effector cells, and increase the immune action against HER2 over-expressing cells. There are also small molecules that inhibit the kinase function of the receptors. Lapatinib is approved in patients with breast cancer, but there are others that bind in a covalent fashion, and they also have remarkable activity.

Finally, there are inhibitors of the Heat Shock Protein 90 (HSP-90). HER2 is a client protein of HSP-90. Blocking HSP-90 function sends HER2 into fast degradation and down regulation from the cell surface.

As mentioned, pertuzumab interferes with receptor dimerization and prevents the formation of both HER2 homodimers and HER2-HER3 heterodimers. It also blocks HER2-HER1 heterodimers, although this heterodimer combination may not be important in breast cancer.

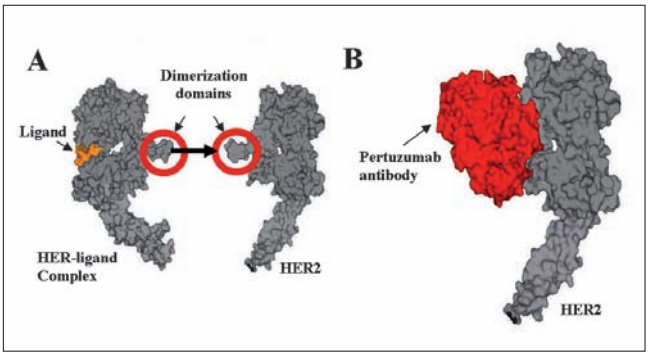

Figure 1
Pertuzumab is a HER dimerization inhibitor.

Pertuzumab was initially developed in patients with HER2-negative disease or with low expression of HER2. The results were not positive as limited anti-tumor activity was observed. On the other hand, in a study of 66 patients with HER2-positive breast cancer who were no longer responding to trastuzumab, the addition of pertuzumab resulted in remarkable clinical activity a clinical response rate, which includes both partial response and total remission, of 25%.

This is a far better result than with any chemotherapy or any other HER2-based drug in a similar patient population. An additional 25% of the treated patients showed disease stabilization (lack of progression of their tumors) for six months or more, giving an overall clinical benefit of 50%[1]. In addition the combination of pertuzumab and trastuzumab, unlike chemotherapy, is not toxic. There are great hopes for this agent and pertuzumab is being studied as first-line therapy in an international registration study of 800 patients.

Like pertuzumab, t-DM1 is safe and does not cause cardiac toxicity. In a phase II study of 30 patients who

had failed trastuzumab therapy, the investigator response rate is 40%. An independent review panel pegs the response rate lower, at 30%, but it is still remarkable.

A novel approach against HER2 is the use of HSP-90 inhibitors. HSP-90 is a chaperone, required for the maturation and stabilization of client proteins. Some of these clients, including HER2, play a major role in breast cancer. The rationale is that blocking HSP-90 will lead to ubiquitination and degradation of HER2.

In the laboratory, treating breast cancer cells with acquired resistance to trastuzumab *in vitro* with an HSP-90 inhibitor such as IPI-504 results in a profound antiproliferative effect. HSP-90 inhibitors are therefore active not only in cells that have not been exposed to trastuzumab, but also in trastuzumab-resistant cells.

Similar responses have been observed in studies with various cell lines. This candidate is being tested in clinical trials in patients who are refractory to trastuzumab.

Clinical aspects

There are a number of practical considerations for clinically developing the agents mentioned above. These include regulatory complexities in the clinical development of novel agents, the changing biology of the tumors over time, and the importance of combining these agents with conventional therapeutics including chemotherapy.

Clinical investigators face a number of challenges in the clinical development of agents that target HER2. First, the standard approach for new drug approval involves the addition of the new therapy to the approved standard of care. However, there are standards of care that result only in minimal improvements in clinical benefit, improvements that are limited to few weeks of progression-free survival and a lack of overall survival benefit. The drug-development process should instead only consider as a standard regimen those candidates that confer a major survival benefit.

Another hurdle is that a tumor's biology is not stable over time, requiring that the tumor may need to be re-interrogated (that is, re-biopsied) as it progresses. Many clinical centers involved in breast cancer research repeat biopsies to try to characterize the tumor's features on a real-time basis. However, this is not easy. Non-invasive methods — such as analyzing the circulating tumor cells — that truly reflect what's happening in the tumor would be a welcome addition and would hopefully reduce the need for invasive tumor biopsies.

Although these agents are quite active, their activity is markedly enhanced when they are combined with chemotherapy. Therefore, a parallel line of research is to continue to improve the chemotherapy agents that are combined with anti-HER2 targeted agents.

For example, when liposomal anthracyclines, which are cardiac safe, are combined with paclitaxel and trastuzumab for patients with either metastatic or locally advanced disease, they generate a tremendous response — as high as 98% in a trial just published[2]. The complete response rate is 50%, so this may be the HER2-positive study with the highest response rate.

To follow up on this data, a large phase III registration study comparing the standard therapy to the experimental arm containing liposomal anthracyclines has been completed, and the data should be available within the next few months.

Drug resistance

Primary or acquired resistance to trastuzumab is one of the limiting factors in the success of anti-HER2 therapies. There are a growing number of well identified mechanisms of resistance against anti-HER2 agents.

One important mechanism of resistance to trastuzumab is the presence of truncated HER2 receptors. About

one-third of patients with HER2 amplified tumors co-express a truncated receptor that has the transmembrane and intracellular domains but is lacking the extracellular domain. Those tumors do not respond to trastuzumab because trastuzumab cannot bind to them.

There are several mechanisms of production of these truncated receptors, collectively known as p95HER2 C-terminal fragments. Proteolytic shedding by matrix metalloproteases results in the generation of receptors that lack the trastuzumab-binding extracellular domain.

However, new data strongly suggest that the more frequent mechanism of generation of these fragments is by an alternative initiation of translation. Alternative translation explains why there are multiple forms of these C-terminal fragments, including the 611 form and the 687 form, frequently co-existing in the same tumor.

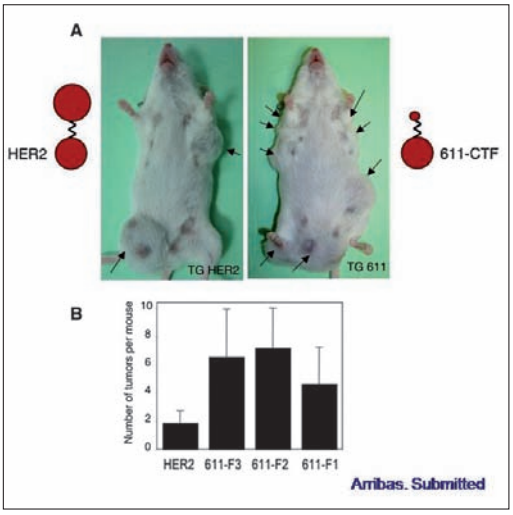

Figure 2
Transgenic mice expressing p95HER2 in the mammary gland.

These forms have the transmembrane portion and are very active. They result in more invasive tumors that grow faster, and transgenic mice expressing p95HER2 in the mammary gland develop more aggressive tumors than in the well-characterized transgenic mice expressing full-length HER2. A study from patients in clinical trials shows that tumors with the p95 receptors respond much less to trastuzumab when compared with tumors expressing the full-length receptor[3].

Only one p95-positive patient in the study responded to the drug. In contrast, those tumors respond to a tyrosine kinase inhibitor such as lapatinib. Hence, tumors with the full-length HER2 receptor respond to both trastuzumab and lapatinib, but those with p95HER2 only respond to lapatinib. p95HER2 is a client protein of HSP-90, so these tumors may also respond to HSP-90 inhibitors.

Lessons from the p95HER2 story suggest that different anti-HER2 agents may have partially non-overlapping mechanisms of action, and that the combination of these agents could potentially yield an enhanced clinical benefit.

Synergistic combinations

In some situations, antibodies and small molecules with different mechanisms of action can be combined. These combinations have proven to be highly synergistic in xenografts and clinical models.

In one study of patients with advanced HER2-positive tumors, the patients progressed on trastuzumab. When they were randomized to receive lapatinib alone versus a combination of lapatanib and trastuzumab, the median time for progression-free survival went up from 8 weeks to 12 weeks with the combination.

The combination is being tested in patients with newly diagnosed HER2 positive breast cancer and prior to surgery (also known as in the neo-adjuvant setting) in a large European study of 450 patients (the NeoALTTO study).

The study also reflects a smarter clinical trial design. Patients with newly diagnosed HER2 breast cancer are given six weeks of biological therapy only. All the patients have mandatory tumor biopsies and optional PET scans at week 2 in order to try and identify biomarkers of clinical benefit even before they become apparent in other ways. The patients are then given chemotherapy for a total therapy time of 18 weeks, followed by surgery.

The trial's primary endpoint is the proportion of patients who do not have the tumor at the time of surgery. After surgery, patients receive more conventional chemotherapy, followed by up to one year of anti-HER2 therapy, because the secondary endpoint is disease-free survival. In this study, particular attention will be paid to the benefits from therapy in patients who express p95HER2.

Another important mechanism of resistance is through deregulation of the PI3K/mTOR/Akt pathway, downstream from HER2. Patients who have PTEN deletions or PI3K activating mutations respond less well to trastuzumab. In a genome-wide shRNA screen, PTEN comes up every time as a potential modulator of lapatinib sensitivity.

There's some hint that mTOR inhibitors may play a role in breast cancer therapy. In a neo-adjuvant study, when patients are randomized to letrozole alone or to letrozole and the mTOR inhibitor everolimus, those given the combination do better by clinical parameters, by ultrasound and, interestingly, by Ki67, a superb biomarker of efficacy in hormone-sensitive disease[4].

By day 15, the highest proportion of patients with low levels of Ki67, or low proliferation, is the group of those that receive the combination of everolimus and letrozole. Proliferation at day 15 also correlates with clinical outcome: those who see the biggest decrease in Ki67 are the ones that achieve the most clinical benefit.

Surprisingly, everolimus also seems to be effective in patients who are resistant to paclitaxel and trastuzumab. A phase II study is trying to confirm those promising data.

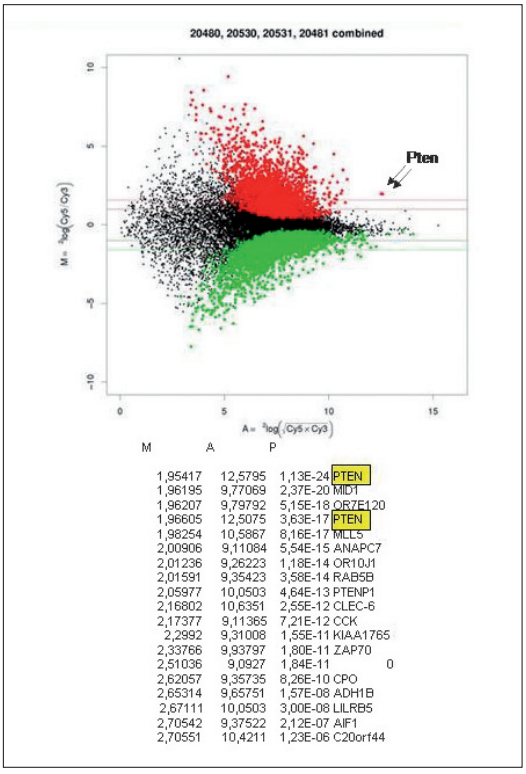

Figure 3
Genome wide shRNA screen identifies PTEN as a potential modulator of lapatinib sensitivity.

Feedback loops

Activation of feedback loops is increasingly being recognized as a potential mechanism of resistance to targeted therapies. For example, everolimus increases the levels of phospho-AKT in patients. Presumably, blocking mTORC1 results in the liberation of a negative feedback loop of S6K over IRS1, and that results in the increase in P-AKT. This could have potentially deleterious effects, prevent the antitumor activity of everolimus, and could even result in greater tumor proliferation.

One approach is to try to prevent the activation of this and other feedbacks loops by multiple pathway targeting. For example, in the case of everolimus, an antibody against the IGF-1 receptor might be able to reverse the effect. Indeed, a phase I study with MK-0647, a Merck antibody to IGF1R, shows that the antibody reduces IGF1R expression and inhibits the phosphorylation of IRS-1, AKT and MAPK[5].

In the lab, the combination of mTOR inhibitors and anti-IGFRs is truly synergistic in multiple models. A phase I clinical trial of the mTOR Inhibitor deforolimus and the anti-IGF1R shows indications of clinical activity in patients with breast cancer.

In samples from patients, S6K also appears to regulate a feedback loop that increases ERK signaling. Samples

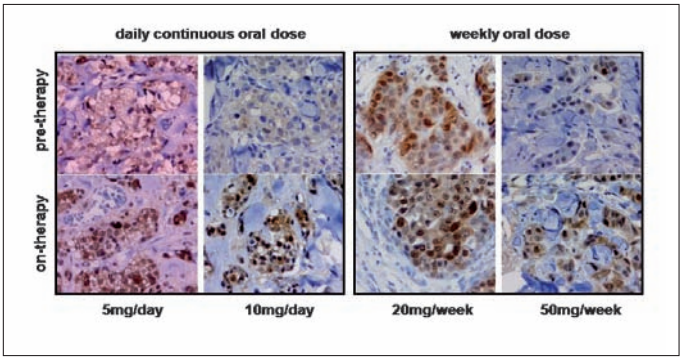

Figure 4
RAD001 increases tumor p-AKT in patients.

from patients have increased levels of both P-AKT and tremendously increased P-ERK activity. The obvious next approach is to try to combine mTORC1 and a MEK inhibition approach. This has been done with multiple inhibitors, and the results are consistent: combined inhibition of mTORC1 and MEK1/2 inhibition induces tumor regression.

There are also many available PI3K inhibitors that are being brought into the clinic. Cell lines naturally expressing or transfected with an activating mutation of PI3K are resistant to trastuzumab, but they are very sensitive to PI3K inhibitors. For instance, the PI3K inhibitor NVP-BEZ235 inhibits proliferation of trastuzumab-resistant cells that over express p110-α mutations.

This is not an absolute resistance, but the cells with this mutation are more resistant than those with the amplification, and more so perhaps than those with the PTEN deletion. Hyperactivation of the PI3K pathway thus regulates trastuzumab and lapatinib sensitivity in HER2 breast cancer cells. Treatment with the PI3K inhibitor NVP-BEZ235 completely blocks growth of these cells with the mutations[6].

In summary, there are inhibitors to HER2, PI3K, IGF1R and others in the pipeline. One important issue is how to develop these multiple lines of therapy for breast cancer. Testing these approaches in patients with advanced disease is likely to give a false negative readout.

In Europe, the Breast International Group has decided to try to study all new agents in the neo-adjuvant model (before surgery) in order to obtain a better readout. The group is also incorporating circulating tumor cells and PET scanning imaging as endpoints. Hopefully, these markers should be able to identify early activity, as observed with everolimus in the neo-adjuvant model.

A more profound implication of these studies is that the underlying genetic alterations for any given tumor should be identified in order to wisely choose the best therapies. In the case of HER2, knowing the amplification status of the gene will not be enough; whether there is presence of truncated forms of the receptor or mutated PI3K is likely to be useful in the selection of the most appropriate therapy.

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PART II: The importance of oncoproteins

Acute promyelocytic leukemia: a model for oncogene-targeted cure

A report on a lecture by
Hugues de Thé
Inserm, CNRS, University of Paris 7, Paris, France

Acute promyelocytic leukemia (APL) is a rare and severe condition characterized by a specific translocation that yields the PML/RARA fusion protein. Retinoic acid (RA) induces both the transcriptional activation of PML/RARA and its degradation, triggering complete remission in cases of APL, and making APL the paradigm for oncogene-targeted therapies. Several Chinese groups have shown that arsenic trioxide can also induce complete remissions in APL. Arsenic also induces PML/RARA degradation, albeit through a completely different pathway than does RA. Used together, the two drugs work synergistically, rapidly clearing leukemia-initiating cells and eradicating disease. Analysis of PML/RARA mutants reveals that differentiation is not the basis for the cure. cAMP also greatly enhances RA-induced APL clearance. These observations exemplify a situation in which cancer therapy was first optimized in the mice and then successfully transposed to patients. ***Hugues de Thé*** *suggested that targeting oncoproteins for degradation could serve as a general strategy for fighting cancers in which the acquisition of stemness results from oncogene expression.*

Acute promyelocytic leukemia (APL) is a cancer of the blood and bone marrow, and a subtype of acute myelogenous leukemia. There is a strong case for oncogene addiction in APL, and available long-term data suggest the possibility of a cure for the disease. It's also an example of a disease for which some research groups have been able to demonstrate the value of using a combination of therapies targeting the same protein.

The first aspect of the APL story unravels the role of PML/RARA degradation. About 20 years ago, a Chinese research group first showed that APL is sensitive to retinoic acid (RA) differentiation therapy. Two weeks after RA therapy, leukemic cells can be seen to be undergoing differentiation.

However, it is important to note that RA alone does not cure APL. An association between RA and anthracyclin has increased five-year survival rates from 25% to 70%, allowing these cases to be classified as "cures". Some rare cases have also been cured with another formulation of RA, which is liposomal RA.

The demonstration of APL's sensitivity to RA led to the characterization of the PML/RARA gene, the product of the t(15,17) translocation. There is strong evidence that, in APL, PML/RARA is the single rate-limiting genetic alteration, making APL a monogenic cancer. One strong piece of evidence in support of that hypothesis is that the incidence of APL is flat with age: a child of 2 years has the same chance of developing APL as a man of 60.

Michael Bishop (see page 37) and others have shown that PML/RARA transgenics develop APL. RA binds to and activates PML/RARA and therefore constitutes a striking example of oncogene-targeted therapy.

Very high concentrations of RA, which overwhelm the system with RA, lead to the recruitment of co-activators, activation of the PML/RARA target gene, differentiation — which was believed to be the origin of clearance of the disease — and clinical remission.

In 1995, two groups including that of Chen Zhu in China introduced arsenic therapy for APL. RA and arsenic appear to share one unexpected property, which is the degradation of the product of the PML/RARA gene.

Separate pathways

When therapeutic concentrations of RA or arsenic are given overnight to primary mouse cells, it is immediately apparent that the mode of action of the two compounds is likely to be very different. Although RA and arsenic can both degrade PML/RARA, they do so through non-overlapping pathways.

RA, through binding to its hormone-binding pocket in RARA, induces a conformational change exposing AF2, recruiting the proteasome and triggering ligand-activated PML/RARA degradation. This has been shown to be a common feature of all nuclear receptors, presumably because they share the same AF2 domain.

Working out arsenic's mechanism is a bit more complicated. Arsenic trioxide triggers degradation of PML/RARA by targeting PML. This catabolic pathway involves an initial SUMOylation of PML or PML/RARA, recruitment of a protein called RNF4, poly-ubiquitination and proteasome-mediated degradation.

Treatment with therapeutic concentrations of arsenic trioxide for one hour induces massive SUMOylation of the PML protein, but only at a single site, K160, of the protein's three SUMOylation sites. This massive SUMOylation is then degraded in a proteasome-dependent manner after 16 hours[1].

There are similar data available for the degradation of PML/RARA. PML is the first and only known substrate of this SUMO-dependent, ubiquitin-mediated catabolic pathway, the existence of which was predicted from many yeast studies.

In order to try and generate an arsenic-resistant APL, PML/RARA mutants are created in which the critical K160 SUMOylation site is replaced with an arginine. Unexpectedly, this residue is absolutely required for transformation.

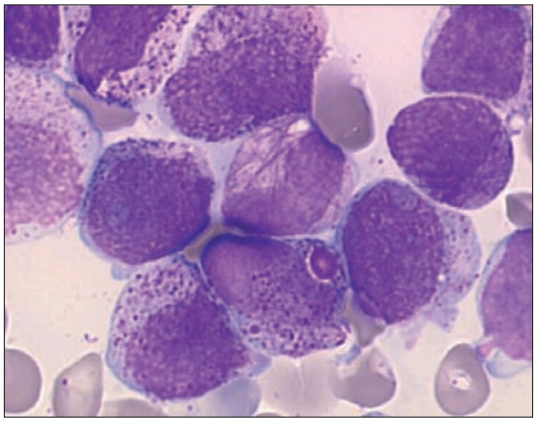

Figure 1
Untreated acute promyelocytic leukemia.

If primary mouse hematopoietic progenitors are transduced using retroviral vectors with PML/RARA, they become immortal and yield a pro-myelocytic phenotype[2]. With the point mutant, in contrast, the cells can be replated three times, which is significantly more than is possible for normal cells, but after that the cells die off. The phenotype is also much less severe in terms of morphology, and the cells don't express immature antigens such as C-KIT.

The K160 residue is able to recruit a critical interactor of SUMO, the Daxx protein. Fusing back the Daxx repression domain to this PML/RARA mutant completely rescues transformation in terms of serial replating, and in terms of C-KIT expression.

This indicates that, in its mono-SUMOylated form, the residue can recruit Daxx and induce transformation. Through a biochemical change that is still being investigated, treatment with arsenic creates a poly-SUMO chain, which then recruits RNF4 and triggers degradation, the basis for its therapeutic effect.

Synergistic effect

In the absence of any treatment, APL inevitably leads to the death of the mice. Treatment with RA or arsenic alone prolongs survival, but treatment with both together eradicates disease, and the mice are essentially cured[3].

These mouse data led to combination trials in patients at three different hospitals in Shanghai, Beijing and Houston. The trials, all of which reached the same conclusion, have shown that the association of RA and

arsenic can cure about 95% of patients. In the case of the trial site at M. D. Anderson Cancer Center in Houston, RA and arsenic were successful, even in the absence of any chemotherapy. This is a critical example of transfer from mouse data to patients, although it took ten years between the two publications.

More recent work has addressed the role of differentiation, and tried to unravel the role of PML/RARA degradation or activation. RA may not have the same effect on the bulk of the APL cells and on the leukemia initiating cells (LICs).

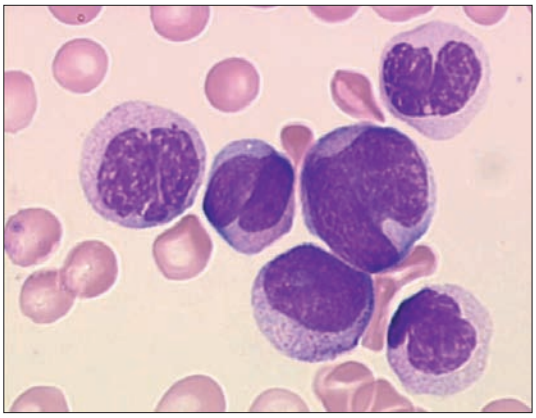

Figure 2
Differentiation therapy by retinoic acid.

If 10,000 cells are transformed with PML/RARA using a retroviral transfection assay, they give rise to 100 colonies. These cells show some asymmetry: despite the fact that they all have leukemic features, only 1 in 100 has stem cell-like or clonogenic properties. When the cells are grown in the presence of RA, there is a dramatic reduction in the number of colonies from 100 to 8, indicating a clear decrease in clonogenic activity in response to RA.

There is also terminal differentiation of those colonies that manage to arise. Surprisingly, if these granulocytes are regrown in the absence of RA, they retain full clonogenicity, suggesting that their clonogenic activity had been transiently blocked, but not destroyed.

In fact, these cells can be grown three times in a row in RA and maintain their clonogenic activity upon removal of the drug. This implies that differentiation and loss of clonogenic activity can be uncoupled *ex vivo*, and questions the idea that differentiation is at the origin of the therapeutic effect.

Clinical resistance

There is also evidence for complete decoupling between differentiation and anti-clonogenic effect in PLZF-RARA APL cells, which are clinically resistant to RA. When mice harboring those cells are treated with RA, they are perfectly sensitive to RA-induced differentiation, but there's no disease regression whatsoever.

When the granulocytic marrows are transplanted into secondary recipients, those mice die at exactly the same time as do the primary mice, implying that although there is complete differentiation in this setting, there is no anti-leukemic effect and no effect on clonogenic growth.

This is a compelling example *in vivo* of the uncoupling between differentiation, which is maintained, and the clonogenic activity, which is unaffected.

Low or insufficient doses of RA are known to be associated with clinical resistance in patients. At a low dose of 1.5 mg, rather than the normal 10 mg, RA triggers the same initial differentiation, but has no anti-leukemic effect in mice. Low dose RA also has no effect on the growth of marrow in secondary recipients.

Revisiting the data on RA/arsenic synergy in mice, it turns out that the dramatic synergy is not for differentiation, but only for the elimination of clonogenic cells. In the secondary recipients, there is a dramatic prolonging of survival after only three days of treatment with the combination of RA and arsenic.

When marrows of primary treated animals are transplanted into secondary animals after six days of treatment, arsenic alone has no effect, RA alone has a modest effect, but the association of the two results in a complete elimination of clonogenic cells.

This probably reflects cooperative PML/RARA degradation by the association of these two non-overlapping

pathways of degradation. If mice are treated *in vivo* with RA and arsenic in combination with bortezomib (Velcade), a clinical grade proteasome inhibitor, bortezomib on its own triggers significant differentiation, but dramatically blocks the elimination of leukemic cells by the RA/arsenic combination. In secondary recipients also, the clonogenic activity is restored when proteolysis is inhibited.

These results imply that the eradication of clonogenic cells is a result of treatment-induced degradation of PML/RARA, which is completely blocked by proteasome inhibitors. Overall, the data suggest that the APL cure reflects eradication of leukemic cells by PML/RARA degradation rather than by activation, resulting in differentiation.

Dramatic crosstalk

Many groups have shown that cAMP greatly potentiates RA-induced differentiation and growth arrest of many APL or non-APL cell-lines, including the F9 embryonic carcinoma cells, HL60 and AML. cAMP-activated PKA phosphorylates RARA in a site that is shared with PML/RARA.

To address whether these are parallel or convergent pathways, clinical grade phosphodiesterase inhibitors are used to elevate cAMP levels in the leukemic cells in combination with low-dose RA. The low dose RA doesn't have anti-leukemic effect on its own. But when cAMP is added, the disease doesn't progress, and spleen weight returns to normal.

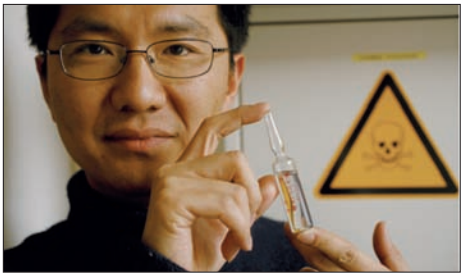

Figure 3
Arsenic, a new therapy for APL.

More important, the ability of the treated leukemic cells to transplant is dramatically diminished with the addition of a phosphodiesterase inhibitor, compared with the sub-optimal RA/arsenic marrows.

This implies a dramatic cross talk between RA and cAMP. To determine whether the effect is mediated through phosphorylation of PML/RARA, a set of transgenic mice are generated in which the phosphorylation site of RAR in the PML/RARA is mutated.

This PML/RARA can still bind RA, but it cannot be phosphorylated by cAMP. It turns out that this leukemia, which has normal APL features, is unexpectedly completely resistant to RA, although it does not show a failure to differentiate[4].

The critical parameter here is not the receptor, which still binds RA and activates target genes. The change is rather that concentrations of RA that normally completely degrade PML/RARA now only partially degrade the phosphorylation site mutant. Thus, this mutant does not show enhancement by cAMP of PML/RARA degradation.

This is also applicable to patients. One patient treated in a St. Louis hospital had undergone every possible regimen, and was completely resistant even to the combination of RA and arsenic. But when the patient was given theophylline, her disease cleared, she stopped being dependent on transfusion and developed a long-term complete remission. She eventually received a bone marrow transplant and is still alive.

Taken together, these results point to a novel view of the pathogenesis of the disease, which includes not just the differentiation block, but the increase in the self-renewal of the LICs.

At the molecular level, in the presence of RA, the differentiation block is de-repressed, most likely through transcriptional activation. But that's not what's curing the disease. Rather, what cures the disease is the arrest of the self-renewal of LICs, which is directly reflected in the level of the PML/RARA protein. All of the agents identified in the past 20 years, including RA, arsenic and cAMP, cooperate to induce synergistic PML/RARA degradation, leading to the eradication of LICs.

Killing leukemia

This is a fantastic story, but one may argue that this is a very peculiar disease, dependent on a single protein. However, unpublished data suggest that it may also be applicable to other settings.

Chronic HTLV-1 infection induces acute T-cell leukemia (ATL), a very severe disease. Recent work has shown that mice expressing the key viral transactivator, the Tax protein, develop an ATL-like disease. Arsenic and interferon-α (IFN-α) trigger a major proteasome-dependent degradation of Tax.

The combination therapy of IFN-α and arsenic, but neither alone, cures many animals in a transplantation model of this Tax-driven ATL. Interestingly, in this case, cure is led by elimination not of the bulk of the tumor, but selectively of the LICs.

In this model, developed by the group of A. Bazarbachi at the American University of Beirut, Tax transgenics show enormous splenomegaly, which is typical for ATL.

Untreated mice die very quickly; those treated by IFN-α or arsenic alone live a bit longer but are never cured. With both together, some of the mice are cured even with an 18-month follow up. A second course of treatment bumps up the cure rate to 80%.

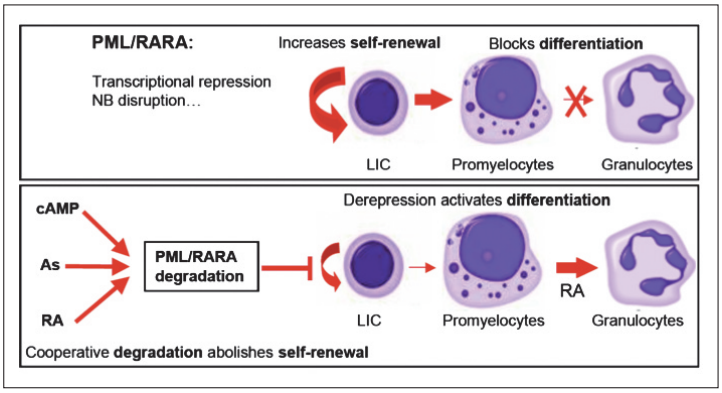

Figure 4
PML/RARA degradation correlates with patient cure.

After treatment for just three days with arsenic, IFN-α, and the two together, there is no significant modification in the tumor bulk. The inoculates are still 100% leukemic, and there is only a modest effect on apoptosis, rising from 10% to 17% under treatment.

However, when these cells are transplanted into secondary mice, there is a dramatic enhancement of survival as a result of treatment with IFN-α and arsenic. This is the equivalent in quantification experiments of an at least 100-fold difference in the LICs driven by a single three-day treatment with agents that degrade Tax, the driving oncogene in this model.

The fact that it is reversible by a proteasome inhibitor is significant indication that it is driven by the degradation of Tax. Adding a proteasome inhibitor essentially blocks the degradation of Tax by IFN-α and arsenic, and eliminates the enhancement of survival in secondary recipients.

Even more strikingly, if leukemic cells from secondary transplants are taken out at a time just before death and transplanted into tertiary recipients, the mice treated with only IFN-α and arsenic all survive, but those treated with IFN-α, arsenic and the proteasome inhibitor all die.

These observations suggest that the treatment does not kill the bulk of the tumor, but suppresses stemness of the leukemic cells, most likely through Tax degradation. The cells can grow, but eventually stop growing and die.

In summary, the combination of RA and arsenic cures APL through the degradation of PML/RARA and LIC eradication, rather than through differentiation. Oncogene addiction is likely to be a general feature of those cancers that are associated with a single genetic or rate-limiting genetic hit, which is the case in many leukemias and sarcomas. Inducing the selective degradation of oncogenes through small molecules could serve as a general strategy to clear cancer stem cells, at least in these single-hit genetic diseases.

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Development of the novel HSP-90 inhibitor IPI-504 and insights into the tumor cell selectivity of HSP-90 inhibitors

A report on a lecture by
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Infinity Pharmaceuticals Inc., Cambridge, USA

Many HSP-90 client proteins are oncoproteins that drive the pathogenesis of different cancers. Elevated expression of HSP-90 has been associated with either disease progression or decreased survival in several types of cancer, making HSP-90 an attractive target for cancer drugs. A novel HSP-90 inhibitor IPI-504 has shown promising evidence of biological activity in gastrointestinal stromal tumors and advanced non-small cell lung cancer. A newly developed assay measures the activity of HSP-90 isolated from patient tissue or mouse xenografts by determining the fraction of HSP-90 molecules that are bound by this inhibitor. This 'occupancy' of HSP-90 correlates with client protein degradation in animal models. Investigating the well-documented selectivity of ansamycin antibiotics for cancer cells versus normal cells shows that 17-AAG has similar affinities to HSP-90 in lysates from cancer and normal cells. However, 'in cellulo' binding of inhibitors to HSP-90 differs between cancer and normal cells and correlates well with cell-growth inhibition. ***Julian Adams*** *explained that the inhibitor's specificity derives from the fact it never binds to HSP-90 in normal cells, or in cancer cells that are insensitive to the drug. However, once normal cells are lysed, the liberated HSP-90 assumes a cancer cell-like, high-affinity state.*

The heat shock protein HSP-90 is one of the most abundant and important protein chaperones, which help in the proper folding of proteins. In oncology, HSP-90 chaperones and folds some of the most nefarious oncoproteins. Inhibiting the chaperone would be expected to induce degradation of those offending oncoproteins (see Baselga, page 17).

About 10 years ago, scientists discovered a natural product called geldanamycin, an alkylating agent far too toxic to develop. But a semi-synthetic analog of geldanamycin called 17-Allylamino-geldanamycin (17-AAG) was tested in more than 400 patients in poorly designed phase I trials.

Because it is insoluble, poorly designed formulations of the drug, including a DMSO-based formulation, caused a lot of initial toxicity. More recently, a nanoparticle suspension has been developed, which may be a better way to deliver this drug.

Still, even though the drug didn't show a lot of activity, it was fairly well tolerated in 400 patients. Conforma, another company working on 17-AAG, showed that 17-AAG is a potent inhibitor of HSP-90, and this inhibition is selective in cancer cells over normal cells[1].

17-AAG's insolubility problem can be solved by making it a salt. If the quinone ring is reduced to a hydroquinone, the hydrochloride salt IPI-504 is about 5000-fold more soluble. The drug can then be delivered via a saline injection, and exposure to neutral pH and oxygen in the plasma then oxidizes it back to 17-AAG[2].

When mice are given IPI-504, they convert it to 17-AAG in the plasma, and also form the primary metabolite, 17-AG, or amino geldanamycin. Oddly enough, giving the mice 17-AAG results in the same pattern of distribution in the plasma, suggesting that IPI-504 and 17-AAG must inter-convert *in vivo*.

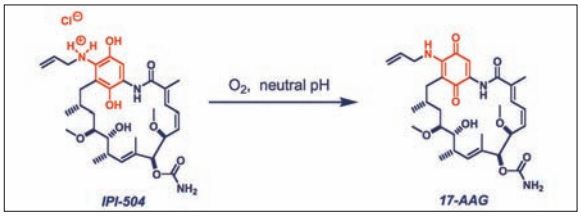


Figure 1
IPI-504 converts to 17-AAG at physiological conditions.

Based on observations in microsomes and a number of different systems and cellular systems, a whole host of NADPH-dependent oxido-reductases can perform the conversion back to IPI-504 *in vivo*.

In experiments with HER2-positive SKBr3 breast cancer cell lines, IPI-504 and 17-AAG both have the same effect, and induce degradation of HER2. Interestingly, the biomarker for inhibiting HSP-90 is the commensurate induction of HSP-70. Again, both IPI-504 and 17-AAG generate overlapping IC50 curves, further confirming that the two drugs act through inter-conversion.

Indirect inhibition

Which of the two is the real drug? In a binding experiment, the binding constant for 17-AAG is about 500 nanomolar. In contrast, IPI-504 has a binding constant of 9 nanomolar, binding roughly 50-fold more tightly to HSP-90 than does 17-AAG.

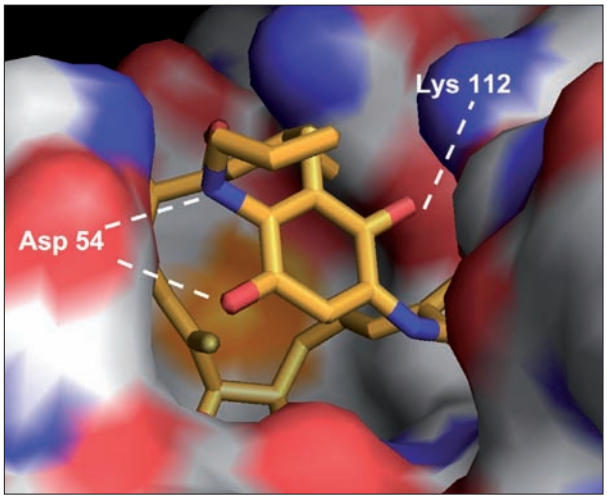


Figure 2
IPI-504 binds roughly 50-fold more tightly to HSP-90 than does 17-AAG.

So, IPI-504 is water-soluble, inter-converts with 17-AAG and is a potent inhibitor of HSP-90. In phase I and II trials, the drug seems to be well tolerated, with biologic and clinical activity, and has been tested for non-small cell lung cancer and HER2-positive metastatic breast cancer[3].

IPI-493, the major metabolite of IPI-504, is an even more active agent, binding at about 3 nanomolar. It undergoes the same inter-conversion, but with a far greater preference for the quinone. When formulated as a molecular dispersion, it has nearly a 100% oral bioavailability, which the Lipinski rules predicted couldn't be done (see Verdine, page 105). This metabolite is being tested in phase I safety trials.

The development of HSP-90 inhibitors offers an alternative to direct inhibition of oncogenic proteins. In the development paradigm, a normal protein somehow acquires a mutation, rendering it oncogenic, and driving cancer cell growth and survival. The protein depends on HSP-90 for proper folding and functioning.

Oral front-line therapies and tyrosine kinase inhibitors inevitably fail because of escape mutations. But these proteins are still dependent on HSP-90 for their function. Introducing IPI-504 in the second- or third-line setting can affect cancer cell death, but the drug could also be combined even earlier with first-line drugs, and perhaps delay the emergence of resistance. Various oncogenic client proteins, including HER2, are being used to test the hypothesis.

In gastrointestinal stromal tumors (GIST), the wild type c-KIT kinase acquires a gain-of-function mutation, rendering it constitutively active and allowing ligand-independent signaling. Upon drug treatment, resistance mutations, particularly those in exon 17, render it "poly" TKI-insensitive.

Comparing an imatinib-sensitive line with an imatinib-insensitive line that has an exon 17 mutation, the GIST cells seem more sensitive to IPI-504. The more resistance mutations there are, the more the cells are dependent on chaperone folding, perhaps making IPI-504 more effective.

In the GIST-882 tumor xenograft, IPI-504 given three times a week induces regression of the tumor. The results are even better in combination with imatinib.

Metabolic coma

The phase I/II trial that followed tested IPI-504 in patients with metastatic GIST refractory to tyrosine kinase inhibitors. GIST is a rare sarcoma that grows primarily in the belly and perineal cavity with liver metastases, and it's very PET-avid. The trial included intensive PET scanning and CT scanning to assess biological activity, which is unique in GIST.

IPI-504 was tested in two schedules: twice-weekly for two weeks with a week off, which is the intermittent schedule; and the continuous schedule, which is less well tolerated. The trial enrolled 45 patients with GIST, and to enhance the safety database, was expanded to include other soft-tissue sarcomas.

The PET imaging proved extremely important. For example, for a patient who progressed on imatinib, sunitinib and eventually nilotonib, the radiotracers in the PET scan are very avid. After four doses of IPI-504, the lights dim, and then return the week off. After three cycles, the standard uptake values for the radiotracer diminish.

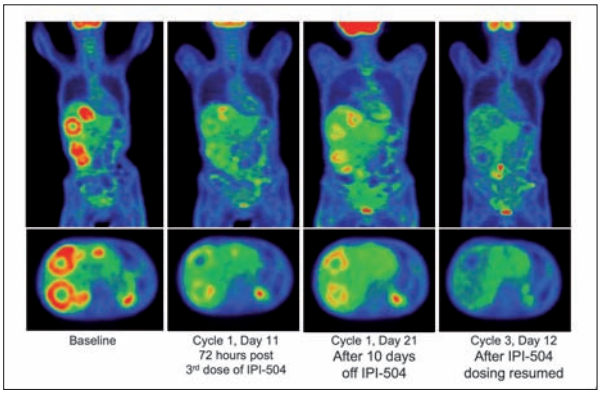


Figure 3
PET imaging of IPI-504 treatment.

The tumor is essentially in a metabolic coma. This is not a function of glucose; glucose transport proteins are not client proteins for HSP-90, although they have been so reported in the literature. These tumor cells are dying, and are not utilizing glucose. Most patients have some sort of stable disease, and many have good responses in terms of PET reductions.

Interestingly, although PET responses improve with subsequent cycles of treatment, but the same lesions imaged by CT show no reduction by RECIST criteria. The observations support the movement in the GIST community that stable disease is meaningful in this disease, and loss of FDG-PET avidity is a marker for biological activity.

The original 147-patient study with imatinib showed that patients who don't respond to imatinib fare poorly. However, patients with stable disease or partial response have the same survival outcome, suggesting that stable disease and partial responses correlate with survival.

In this study, in an analysis of about 30 patients, the disease control rate is about 70%, including a 67% rate of stable disease and 1 partial response. The median progression-free survival is about 12 weeks.

The approval study for sunitinib, a second-line therapy for this disease, is informative. In a study of several hundred patients, the response rate is very low, about 7%. Progression-free survival with sunitinib is 24 weeks, whereas with Gleevec, the first-line therapy, it is about a year and a half.

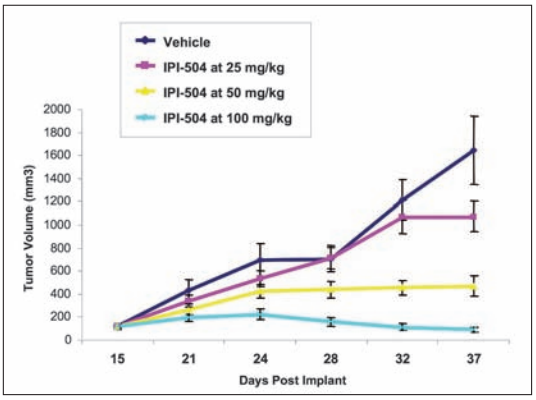
Interestingly, progression-free survival in the placebo arm of this study is between 4 and 6 weeks, so 12 weeks of progression-free survival is probably tripling progression-free survival. The phase III trial, which has a 2:1 double-blind randomization of active to placebo, is open in 16 countries and aims to enroll 195 patients.

Resistance mutations

IPI-504's relevance to non-small cell lung cancer (NSCLC) is much more complicated. Resistance to inhibitors of epidermal growth factor receptor (EGFR) is a big problem.

A xenograft model of NSCLC containing the common T790M resistance mutation in EGFR is not responsive to erlonitib at all, but responds to IPI-504. In combination with gefitinib, IPI-504 is also active in NSCLC xenograft with a MET amplification. MET is a great client protein for HSP-90.

Figure 4
IPI-504 is active against EGFR mutants resistant to tyrosine kinase inhibitors.



An ongoing phase I/II study of IPI-504 in patients with NSCLC included 10 patients with EGFR mutations and 10 patients with wild type EGFR. The patients were given 400 mg/m2 of IPI-504 twice weekly for two weeks, followed by a week off. Of nine patients, seven, or 78%, have at least stable disease by RECIST criteria. One patient with an EGFR mutation has extended survival of six months. The PET responses are also very good.

Waterfall plots of the CT responses show that two patients with wild type EGFR went on to have a partial response, which is surprising. It's not clear what the client proteins are in those cases.

HER2 is one of the most sensitive proteins for HSP-90. There are a multitude of mechanisms of resistance in HER2-positive breast cancer, particularly the presence of the p95 truncated receptor.

In a trastuzumab-sensitive gastric cancer xenograft, IPI-504 is effective alone and in combination with trastuzumab (Herceptin). IPI-504 is also active in trastuzumab-resistant cell lines (see Baselga, page 17). In trastuzumab-sensitive and resistant BT-474 cells, IPI-504 degrades P-AKT and p-MAPK.

In a proof-of-concept trial, 17-AAG shows a 28% response rate against HER2-positive breast cancer in trastuzumab-resistant patients. Follow up trials are enrolling patients.

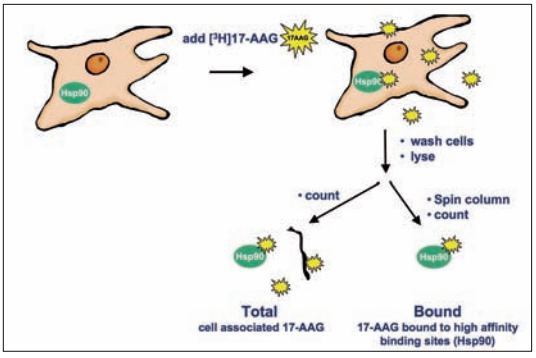
Of all tumors, results in CML are the easiest to analyze. The constitutively active kinase in this case is BCR-ABL, and resistance relies on a T315I mutation. When T3151 BCR-ABL is transplanted into mice, the mice die quickly, lasting only about 20 days.

Imatinib is completely ineffective in this case, but mid- or high-dose IPI-504 confers a tremendous survival advantage to the mice. A combination of IPI-504 and imatinib also prolongs survival in mice that have a mixed transplant of wild type and T315I BCR-ABL.

Assessing occupancy

An occupancy assay can measure HSP-90 activity in these tissues. One thing that is important for the occupancy assay is that the K_{on} has to be much longer than the K_{off}. Fortunately, 17-AAG has a slow off rate, particularly at 40°C, and a half-life of 30 hours, so the cells can be manipulated without the drug dissociating away from its target.

Figure 5
Binding of 17-AAG to HSP-90 inside intact cells.



Unoccupied binding sites are titrated with radiolabeled 17-AAG to calculate the percent occupancy of the free versus bound fractions. When the H1650 NSCLC xenograft is treated with a single dose of IPI-504 at 0, 1, 2, 4, 6, 12, 24, 36, 48 hours, it's clear that the occupancy matches tumor PK better than it does plasma PK.

The plasma PK is uninstructive, and the drug is gone by 6 hours. The tumor PK is a little more interesting. There's still some drug left at 6 hours but by 12 hours, the drug is cleared. In the occupancy assay, the is drug on board at 12 hours, just slightly more than the half-maximal inhibition, so the drug is bound to HSP-90 between 12 and 24 hours.

Correlating occupancy to EGFR degradation, ideally the drug would be given every day or at least every other day. Because it's an intravenous drug, it's being given twice weekly, which is not optimal. An oral version of the drug would afford more flexibility.

A whole tumor xenograft study gives both a dose response and an occupancy response, and shows that the highest occupancy, on the order of 65%, is at the highest dose. It's not clear what the ceiling is on the occupancy.

Ansamycin antibiotics are thought to selectively kill cancer cells over normal cells. According to a 2003 paper, this is because HSP-90 is preferentially complexed with co-chaperones, and this complex has a higher affinity to 17-AAG[4].

But various teams have had trouble reproducing these results, particularly the data on the binding affinity of 17-AAG to HSP-90. In fact, a reconstituted functional co-chaperone HSP-90 complex from purified recombinant proteins has the same affinity to 17-AAG as does purified HSP-90[5].

Lysates from SKOV3, an ovarian cancer cell line, and from normal human diploid fibroblasts have the same binding affinity for 17-AAG.

Another kind of assay, called the *in cellulo* binding assay, measures the binding of 17-AAG to HSP-90 inside intact cells. This assay shows that the SKOV3 cells retain their high affinity whereas normal diploid fibroblasts bind in the micromolar range, suggesting that the in cellulo binding is different in cancer cells than in normal cells.

What's more, that difference correlates with growth inhibition. The most sensitive cell lines — some of the breast cancer and lung cancer cell lines, for example — are also the most sensitive in terms of binding and growth inhibition. However, there are some mixed populations where there is a divergence. To date, there are no known client proteins for certain lymphomas or myelomas.

Overall, these data suggest that cell sensitivity directly correlates with binding of 17-AAG to HSP-90 within cells. This means that normal cells are not insensitive to the inhibition of HSP-90. Rather, the HSP-90 in normal cells is only weakly bound and poorly inhibited. The finding also extends to cancer cell lines with different sensitivities to 17-AAG.

Acetylation doesn't seem to be the basis for the difference, based on experiments with HDAC inhibitors. Other mechanisms that might explain the differences in sensitivity of cancer and normal cells are being investigated, including post-translational modification of the target HSP-90 complexes. The other possibility is that HSP-90 is compartmentalized, meaning that, in normal cells, HSP-90 is not accessible to 17-AAG, and this difference is lost once cells are lysed.

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MYC as therapeutic target: attacking from the flanks

A report on a lecture by
J. Michael Bishop
University of California San Francisco, San Francisco, USA

The proto-oncogene MYC encodes a highly pleiotypic transcription factor that plays a role in diverse cellular functions. Over-expression of MYC is a widespread anomaly among human malignancies, making the protein a tempting therapeutic target. Some tactics that might be useful in treating malignancies utilize synthetic lethal interactions between elevated levels of MYC and inhibitors of either the CDK-1 cell cycle kinase or the mitotic kinase Aurora B. Inhibition of CDK1 elicits rapid and extensive apoptosis of cells, whereas inhibition of Aurora B kinase elicits two forms of death: about one-third of the cells die of apoptosis within three days, and the remaining develop massive autophagy, suffering a more delayed death. Glutamine metabolism may also serve as a potential locus for therapeutic targets against tumors that over-express MYC, because many cancer cells consume glutamine at a voracious rate. A fourth tactic is directed at human lymphomas that owe their genesis in part to antigen-stimulated signaling from the B-cell receptor, and that over-express MYC. ***Michael Bishop*** *explained that, in at least some instances, treatment outcomes for cancer can be improved by directing two distinctive therapeutics at the same molecular target — in this case, two or more synthetic-lethal effects that each exploit the over-expression of MYC.*

The proto-oncogene MYC encodes a highly pleiotypic transcription factor. MYC plays a role in the activation and repression of transcription of about 10,000 genes. It's involved in a variety of normal cellular process including cell cycle, cell growth, cell size and differentiation, and apoptosis when required.

When the gene is anomalously over-expressed, it can transform cells in culture and give rise to tumors in transgenic animals. Anomalous over-expression of MYC is among the most common genetic abnormalities in a wide variety of human cancers, making MYC an obvious target for drug development.

However, MYC has generally not been viewed as a good target. First, it is the wild type gene that is usually the culprit, so there is no therapeutic hook. Second, MYC has also long been thought to be essential for normal cells, so any inhibition of the protein is likely to be toxic. And third, as a transcription factor, MYC has been viewed as a poor target for inhibition by small-molecule drugs.

Cyclin-dependent kinases (CDKs) are members of a family of kinases that are involved in the regulation of the cell cycle. Inhibitors of CDKs have been pursued as therapies for cancer. The drug purvalanol shows reasonably high specificity and activity against CDK1, and leads to G2 arrest in the cell cycle. This arrest is reversible. The cells can be kept in an arrested state for three or four days, and recover when the drug is withdrawn.

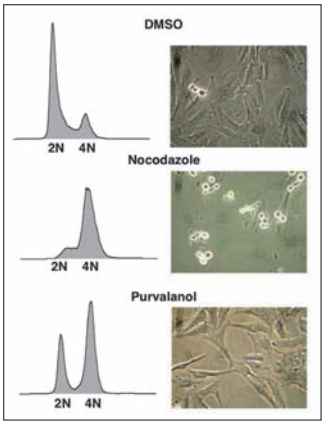

Figure 1
Purvalanol triggers G2 cell cycle arrest.

However, there is an exception to this. Any cell that over expresses MYC — whether a tumor cell or a normal cell that's ectopically expressing MYC — responds to purvalanol with prompt apoptosis. This is a MYC-specific effect that can also be demonstrated *in vivo* with a model for liver cancer, a primitive tumor known as hepatoblastoma. This is a MYC-driven tumor, very aggressive and rapidly lethal, and the transgene is under the control of doxycycline.

In the simple protocol, three weeks after the transgene is activated, the control liver is riddled with tumor. In contrast, the liver of an animal that has been treated with purvalanol looks nearly tumor-free. There are microscopic foci of tumors, and the animal will eventually die, but the prolongation of life is remarkable after just a single short course of therapy. There are similar data in a MYC-driven early B-cell tumor.

Over-expression of MYC may serve as a biomarker for tumors that are susceptible to inhibition of CDK1[1]. What's more, the reversible arrest of healthy cells, which express normal levels of MYC, offers hope that pulse regimens may limit toxicity.

Surviving apoptosis

Survivin is one of the primary components of the Chromosomal Passenger Protein Complex. The complex also includes a protein called INCENP and a kinase called Aurora B, which is the therapeutic target.

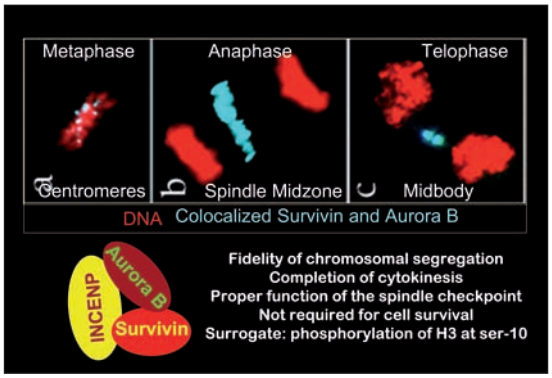

Figure 2
The chromosomal passenger protein complex.

The complex has diverse duties, including helping sustain fidelity of chromosomal segregation, completion of cytokinesis, and proper functioning of the spindle checkpoint. Inhibition of Aurora B kinase arrests the cell cycle both at the G1/S transition and in cytokinesis.

VX-680, an inhibitor of the Aurora B kinase, triggers biphasic killing in cells. There is immediate apoptosis of about 30% of the cells over-expressing MYC, but no killing of the counterpart normal cells.

When human retinal epithelial cells are made to artificially over-express MYC, they become susceptible to a synthetic-lethal interaction between inhibition of Aurora kinase B and over-expression of MYC.

This experiment has been repeated in a large variety of cells, and the limit has stayed at about 30%, which is puzzling. However, it has since become clear that the rest of the cells that over-express MYC do die later, and they die even after the drug is removed[2]. Normal cells recover from the arrest of cytokinesis and resume growing.

The immediate death is a result of apoptosis. Delayed death is also independent of p53 and involves the induction of Bim. Surprisingly, however, the cells that die after a delay are polyploid and highly vacuolated and, by electron microscopy, they contain phagosomes. These cells appear to die as a result of autophagy, which is accompanied by induction of autophagic genes, ATG 6, 8 and 9.

Over-expression of MYC is responsible for this induction. If the cells are rendered deficient in any of these genes — either by RNAi or, in two instances, with genetically deficient cells — the autophagic response to VX-680 and delayed death attenuates by about 80%.

Comparing across a variety of tumor cell lines, cells that over-express MYC are much more likely to display the immediate death response than those that do not over-express MYC. The same sort of trend is seen with delayed death.

Celubrious paradox

MYCN, a cousin of MYC, is amplified in some of the more aggressive forms of human neuroblastoma. When the gene is not amplified in stage IV neuroblastoma, the response to treatment is excellent, so MYCN is

now a standard biomarker in the management of the disease.

MYCN represents a celubrious paradox in this case: if the gene is amplified, the prospects for contemporary therapy are dismal. However, the response to VX-680 is both immediate and delayed toxicity of the tumor cells.

If the gene is not amplified, there's neither acute killing nor delayed killing of tumor cells. So, the amplification, which is a dire prognostic marker, may also be a biomarker for sensitivity to inhibitors of Aurora kinase B.

A MYC-driven T-cell lymphoma is used to demonstrate dependence of tumors on the initiating oncogene. The disease is very aggressive in mice, but a single course of treatment with VX-680 extends the lifespan. It hasn't yet been proven whether this is a reflection of over-expression of MYC in these tumors.

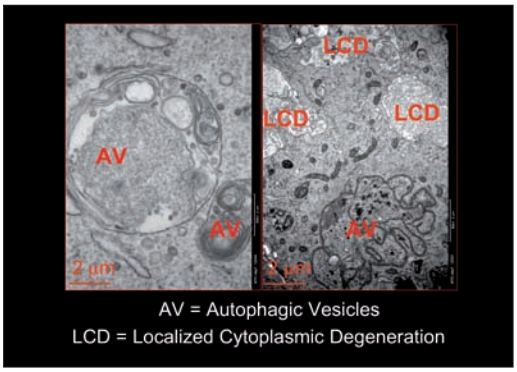

Figure 3
Autophagy accompanies delayed death in response to VX-680.

Repeated treatments can extend the lifespan, but the animals eventually do die, either because the tumors bypass MYC, or develop resistance to the drug.

The mechanism of this synthetic-lethal interaction is that VX-680 exerts its effect by arresting cytokinesis, which induces the p53 checkpoint for DNA synthesis. MYC blocks the induction of this p53 checkpoint — or, in some tumors, the checkpoint is intrinsically defective — and the cells indulge in illicit DNA synthesis, resulting in polyploidy and multi-nucleation.

MYC then mediates immediate apoptosis, which can be demonstrated using RNAi; knocking down MYC blocks this apoptosis. MYC also induces ATG genes in the surviving polyploid cells, and this is followed by autophagy, leading to delayed cell death.

Over-expression of MYC may well be a biomarker for exceptional sensitivity to VX-680. Because of the delayed death during which normal cells would be recovering, a pulsed application strategy, which may spare normal cells and minimize toxicity, seems promising.

Intermediary metabolism

Many cancer cells use glucose inefficiently, through glycolysis rather than through oxidative phosphorylation. Both normal and tumor cells die when deprived of glucose. Tumor cells typically are also voracious consumers of glutamine.

There might be a synthetic lethal effect between over-expression of MYC and the depletion of glutamine because among the many targets of MYC, there are quite a few that play a role in intermediary metabolism.

Deprivation of glucose leads to cell death irrespective of whether MYC is on, over expressed or not. In the case of glutamine, however, in cells that do not over-express MYC, glutamine depletion arrests the cell cycle; the cells recover when glutamine is restored. In cells that do over-express MYC, as many as 80% of the cells promptly die by apoptosis[3].

This is a glutamine-specific effect. Systematically depriving the cells of any of the other amino acids doesn't result in the same synthetic-lethal interaction. Although the exact mechanism of the response is unknown, it doesn't seem to involve arrest of the cell cycle, deficiency in protein synthesis, nucleic acid synthesis or ATP, accumulation of reactive oxygen species or DNA damage.

What is known from metabolomic analysis is that when the cells are deprived of glutamine and they're over-expressing MYC, it strips the Krebs cycle clean so that it's barely functioning. Driving the Krebs cycle with

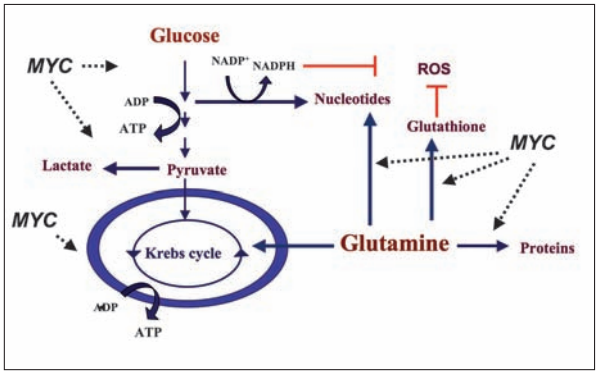

Figure 4
Intermediary metabolism as a keyboard for MYC.

either pyruvate or oxaloacetate rescues the synthetic-lethal effect. It's unclear how the stripping of the Krebs cycle is involved in apoptosis, however.

To assess these responses *in vivo*, three models of liver cancer are used. MYC gives rise to the hepatoblastoma, MET gives rise to hepatocellular carcinoma and RAS alone gives rise to an aggressive anaplastic tumor.

DON, or 6-diazo-5-oxo-l-norleucine, is a small molecule that inhibits glutaminase and transamidases; 20767 is a specific inhibitor of glutaminases. DON was originally an antibiotic, and is a very effective inhibitor. DON displays a synthetic-lethal interaction with over-expression of MYC, killing cells that have high MYC levels. This killing is not extreme in cells that express normal levels of MYC. It is important to note that DON has not been proven suitable for use in humans because of toxicity: it is less specific than the experimental inhibitor used in the next set of experiments.

The glutaminase-specific inhibitor appears to be relatively gentle on normal cells, and only kills cells that over-express MYC. Based on the metabolomic analysis of the MET and RAS models, it is possible that it will be effective even in tumors that don't over-express MYC.

In conclusion, glucose deprivation appears to be a relatively blunt weapon. In contrast, specific inhibitors of glutaminase may be useful against any tumor that requires glutamine for intermediary metabolism.

Suppressing immunity

Inspired by decades of accumulated evidence that some B-cell lymphomas may be a result of autoimmunity, a lymphoma model has been developed with an auto-antigenic loop in cells that express MYC in the B cell lineage.

What has emerged from this work is an animal model for Burkitt lymphoma. MYC in this context suppresses immune tolerance, with a consequent immune response to the auto-antigen. This leads to the early onset of very aggressive lymphoma with characteristic anatomical distribution, pathognomonic histopathology and surface immunophenotype of Burkitt lymphoma or other very aggressive large-cell lymphomas. These tumors remain dependent upon both the MYC transgene and the auto-antigenic stimulus. If either is interrupted, the tumors melt.

The most dramatic anatomic feature of these mice is the classic presentation of jaw tumor that Burkitt encountered. These tumors respond to immunosuppressants in a way that other B-cell tumors in the same mice do not.

Cyclosporine A, FK506 and rapamycin all have a dramatic effect on the tumor in simple transplant experiments, for example. But in a different model that is not dependent on auto-antigenic stimulus, the cells do not respond uniformly to the various immunosuppressants[4].

Although these drugs act in ways that might not involve immunosuppression, it is remarkable that of all the B-cell models examined, only these respond uniformly to a variety of immunosuppressants. The responses are remarkably durable. Many of the animals live a normal lifespan after a full course of treatment with one of the immunosuppressants.

In conclusion, work discussed in this presentation derives from the fact that over-expression of MYC is pro-apoptotic. This capability must be circumvented to facilitate tumorigenesis. Various circumstances that stress tumor cells apparently can uncover the latent pro-apoptotic effect of MYC and, in some instances, promotion of autophagy.

This synthetic-lethal effect may offer a therapeutic alternative to agents that target the MYC protein directly, emphasizing MYC as a potential biomarker for sensitivity to certain drugs, such as inhibitors of CDK1 and Aurora kinase B.

The diversity of synthetic-lethal interactions with MYC might lend themselves to permit synergistic combination therapy. The caveat is that, superficially, it appears that the CDK1 interaction and the VX-680 interaction may be acting through the same apoptotic pathway.

That may undermine the virtue of combination therapy with those two particular proteins, albeit not completely because VX-680 also induces autophagic death, and in some human cell lines, it does that in the absence of inducing apoptosis.

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PART III: Harnessing the immune system

Immune checkpoint blockade in cancer therapy: new insights and opportunities

A report on a lecture by
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Memorial Sloan-Kettering Cancer Center, New York, USA

Clinical strategies to mobilize the immune system against cancer cells have so far been disappointing. One reason for this is that, until recently, insufficient attention was paid to the complexity of the regulation of T cell responses. The prototype of the cell-intrinsic inhibitory pathways is the CD28 homolog CTLA-4, which limits T cell proliferation. Blocking CTLA-4 with a monoclonal antibody, either by itself or in combination with other agents, can greatly enhance anti-tumor responses and long-lived immunity in a number of experimental tumors in mice. In clinical trials, anti-CTLA4 has generated objective, durable responses in about 15% of patients. These responses appear to correlate with pre-existing or induced high-titer antibodies to the antigen NY-ESO-1, and to sustained high levels of the activation marker ICOS. Results with anti-CTLA-4 have generated interest in additional inhibitory members of the extended B7/CD28 family. Finally, the genetic instability inherent in cancer results in an extraordinary number of coding mutations in cancer, many of which give rise to neo-antigens. ***James Allison*** *suggested that combinations of conventional therapies with immune checkpoint blockade may effectively treat cancers and minimize the chances of tumor resistance and escape.*

Scientists have been trying to vaccinate and mobilize the incredible power and specificity of the immune system against cancer for decades, but with little success — despite knowledge of the molecular targets that T cells recognize in human tumors.

One of the main factors for the lack of progress is the failure to recognize the complexity of regulation of the immune system, particularly of the T-cell system. T-cell responses are so powerful that there have to be negative circuits built in to turn them off, limit their expansion or cause them to die, and prevent them from destroying normal tissue. These checkpoints frustrate the ability to successfully mobilize the immune system.

The main focus for the past dozen years has been on events that occur very early in the immune response. A T cell receives a signal through an antigen receptor and then needs a contemporaneous co-stimulatory signal through CD28. This results in the up regulation of cyclins, pRb and the production of interleukin-2 (IL-2), and by down regulating $p27^{kip}$ allows the cell to progress through the cell cycle.

It also induces anti-apoptotic factors, which keep the T-cells alive, and transcriptionally induces production of CTLA-4, a close relative of CD28. CTLA-4 binds the same ligands as does CD28, but it has an off signal. So as CTLA-4 accumulates, it titrates down IL-2, which keeps $p27^{kip}$ up, and slows down the cell cycle.

This down regulation provided by CTLA-4 is a very important regulator of immune responses. CTLA4-deficient mice turn into giant lymph nodes by the time they are 3 weeks old because their T-cells become spontaneously activated and infiltrate every tissue.

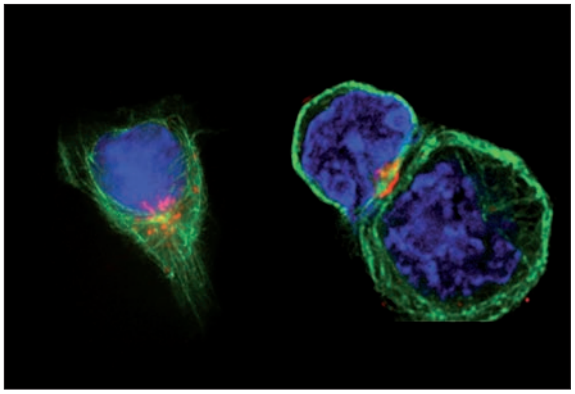

Figure 1
Localization of CTLA-4 to the T cell-APC interface.

CTLA-4 is separated from CD28 not just in time, but also in space. CD28 is constitutively expressed and is always on the cell surface. Within seconds of a T cell hitting an antigen-presenting cell (APC), CD28 starts accumulating at the synapse. That happens after the calcium pulse that follows T-cell receptor signaling but it's a fairly proximal event.

On the other hand, even after CTLA-4 is made, it is retained in vesicles that are tethered to the microtubule-organizing center inside the cell. The molecule is inside the cell and has to be dragged through the cytoplasm as the cell polarizes, comes into apposition at the contact point with the APC, and is literally sprayed into the synapse and begins to down titrate co-stimulation from CD28.

If CTLA-4 limits immune responses, it might also limit anti-tumor responses. Up regulation of CTLA-4 could attenuate or perhaps even terminate responses.

Once a T cell is induced after seeing a viral antigen, the clone is expanded and the T cells effectively eliminate virus-infected cells. The CTLA-4 protein has a half-life of about an hour and a half, so once they disappear, T cells are ready to react again.

But with something like a large tumor mass, the T cells keep hammering at it every time they get a signal, with or without an accompanying CD28 signal. The cell then constitutively expresses CTLA-4 and limits its response.

Antibodies to CTLA-4 take these brakes off the system, and allow for a full realization of T-cell proliferation without the constraints that are normally imposed, making it possible for them to go on and destroy tumors[1].

This could be used as a monotherapy to take advantage of inflammatory tumor cell death, or in combination with vaccines to enhance their potency, or with almost anything that kills tumor cells, including targeted therapies. Because this is a molecular component of the immune system, this is a therapy that's potentially universally applicable to any kind of cancer.

Killing combination

When a colon carcinoma tumor is grown on the backs of mice, blocking CD28 causes the tumor to grow faster, suggesting that CD28 tries to limit tumor growth in some way. In contrast, blocking CTLA-4 causes the mice to reject the tumor, which is remarkable.

These mice are permanently immune to re-challenge with the tumor. The effect requires the immune system because depleting CD8 or CD4 T cells before the experiment has no effect.

The approach doesn't always work and, in fact, against most poorly immunogenic tumors in mice, anti-CTLA4 on its own has no effect.

A commercially developed GM-CSF cytokine tumor cell vaccine system (Gvax) can prophylactically immunize against subsequent challenge with B16 melanoma, but doesn't do anything if the tumor has already been present for a week.

However, when the vaccine and the CTLA-4 antibody are combined, zero plus zero equals 100%, totally curing the tumor. The same immune lessons again apply, meaning that this requires both CD4 and CD8 T cells. Treatment with both anti-CTLA4 and Gvax also activates the vasculature, allowing T cells to extravasate into the tumor and begin destroying tumor cells.

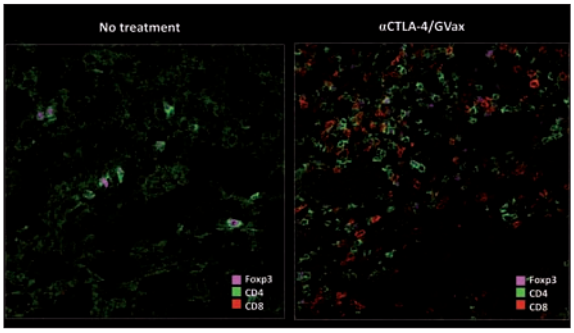

Figure 2
aCTLA-4/GVax increases number of tumor infiltrating T cells.

The kind of T cells present is important. In the absence of treatment, two weeks after tumor implantation, there are virtually no CD8 killer cells and a few CD4-positive cells, many of which have nuclear staining for FOXP3. FOXP3 is a transcription factor that denotes suppressive T regulatory cells that secrete TGF-β, and IL-10, and shut down everything else.

Treatment with anti-CTLA4 generates a lot of CD8 cells and CD4 cells that are negative for FOXP3 expression, and quickly eliminates the tumor. This same effect is also seen in people. By array analysis, there is an increase in the expression of γ-interferon and TNF-α and a decrease in TGF-β and IL-10. These changes essentially transform the environment into a permissive one[2].

Apart from GVax, CTLA-4 also synergizes with most anything that kills tumor cells or primes T cells, including peptide-pulsed dendritic cells, DNA vaccines, depletion of T regulatory cells and adoptive T cell transfer. It can also work in concert with conventional therapies, such as cisplatin, local irradiation, androgen deprivation, surgical reduction and, more recently, cryoablation of poorly immunogenic tumors.

Response rates

A CTLA-4 antibody, originally called MDX-010, and named ipilimumab by the Food and Drug Administration, blocks binding of CTLA-4 to CD80 and CD86, and augments immune responses in primate models.

So far, the antibody has been used to treat more than 4,000 patients in various trials. The large majority by far of these patients have late-stage melanoma, although there are also a few hundred with renal cancer, about 100 with prostate cancer, and a dozen or so with ovarian cancer. There have been objective responses in all those types of tumors.

Overall, by RECIST and modified World Health Organization (WHO) criteria, the response rate in stage IV, heavily pre-treated melanoma is about 15%. Studies in prostate cancer, consistent with mouse studies, suggest that the response rate is higher when combined with other therapies. The responses are durable, and last for years without retreatment. For instance, one woman treated in a phase I trial six years ago had a complete response and is still disease-free.

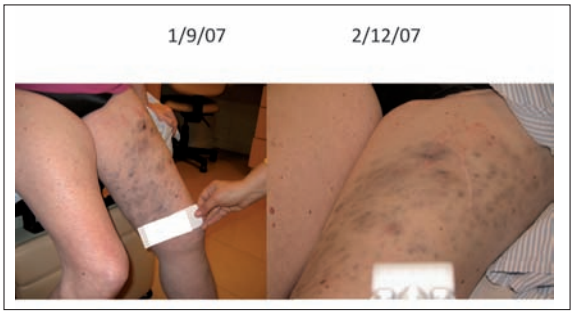

Figure 3
The responses with ipilimumab last for years without retreatment.

However, adverse events associated with the antibody can be serious, particularly when it is given frequently. The primary side effect is colitis. In prostate cancer, there are also some cases of hypophysitis, but these typically resolve with symptomatic treatment and cessation of therapy. When doses of the antibody are spaced out by two months or more, the side effects are limited to mild skin rashes.

The response rate as assessed by RECIST underestimates the real magnitude of the response. In one patient with cutaneous lesions of melanoma, for example, six weeks after the baseline in November 2006, the tumors had largely receded. About 10 weeks after that point, the tumors had completely disappeared. By histology, there were no viable tumor cells left, and there was melanin deposited by the dying tumor cells.

What is evident in these sites where the tumors had been is that there is abundant infiltration by both CD4- and CD8-positive T cells, consistent with previous results in the mouse.

In one particular case, tumors looked prominent at the time of initiation of therapy. About 12 weeks into the trial, at the evaluation point, the tumors were bigger and there was a new lesion, so by the modified WHO criteria, the patient was considered as having progressive disease and was placed in the 'no response' category.

However, about two weeks after that, the tumors were mostly gone and two more weeks after that, the tumors had largely disappeared. Little over a year later, the tumors were still present, but the disease was stable. So, what was considered a failure was actually close to a complete response.

In another example, a patient with extensive liver metastases was treated. At 12 weeks, he also had progressive disease, and was placed into the no-response category. But he felt great, his night sweats were gone, and he could sleep through the night. About eight weeks after that, his tumors had mostly regressed and by week 36, they were essentially gone.

These cases suggest that the RECIST criteria don't always apply, so it may be better to instead look at survival.

Prolonged survival

When patients are given first-line DTIC therapy, at the end of a year, only about 30% of patients are alive, and that number drops to between 10 and 15% at two years. In contrast, with the lowest dose of anti-CTLA-4, there is a 30% survival at the end of two years, and it takes four years for that to drop to 10-15%. At higher doses of the antibody, the survival is much higher, reaching close to 60% at the end of the first year.

Taken together with the fact that about 10% of the patients have disease control after progression, the overall response rate in terms of survival is about 35%. This is a fair way of looking at the numbers because they correlate with prolonged survival, which is the ultimate goal of these therapies.

In March 2003, an 81-year-old woman with disseminated ovarian cancer was given an autologous GM-CSF transduced vaccine made from her own tumor cells. The vaccine on its own did nothing. She then got a single dose of anti-CTLA4, and the levels of CA125, the ovarian cancer marker, dropped. When her disease recurred, she was given another dose, and that cycle has since repeated for six years, with her disease being managed by occasional shots of anti-CTLA4.

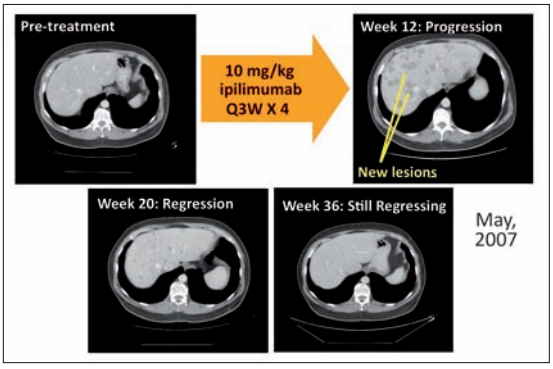

Figure 4
Ipilimumab pattern of response.

For further clinical development of anti-CTLA4, researchers will need to first resolve the cellular and molecular mechanisms involved in the anti-tumor effect in people, the factors that distinguish responders from non-responders, and the best conventional therapies or vaccines to be used in combination with the antibody.

The handful of patients who are objective responders and the bigger group of patients who are not offer a golden opportunity in tumor immunology. Comparing the two groups can help identify the factors that make them different.

One promising lead is NY-ESO-1, a highly immunogenic antigen expressed by about 40% of melanoma cells. Of eight responders, five had either pre-existing or induced antibody titers to this molecule, compared with none of the non-responders. The presence of antibodies to this antigen suggests the existence of prior immunity, predisposing towards a favorable outcome for subsequent treatment with anti-CTLA4[3].

When these antibodies to NY-ESO-1 are present, the responses are typically CD4 and CD8 T cell responses. There are cells that produce both γ-interferon and MIP-1 β, as well as those that produce γ-interferon and TNF-α. In patients that do not respond, T cells that produce cytokines typically make only a single cytokine, but in the responders, the cells tend to be poly-functional, producing multiple cytokines.

Objective marker

As part of a pre-surgical neo-adjuvant antiCTLA-4 trial in bladder cancer, patients are given two doses of anti-CTLA4 before they go to cystectomy. Blood and tumor tissue from these patients could help obtain a clearer picture of the response.

One factor that is immediately noticeable is a big increase in the number of cells that express very high levels of a molecule called ICOS. About 13% of cells in untreated normal tissue and 16% in untreated tumor tissue express this molecule, but the number jumps to 40% in tumors treated with anti-CTLA4.

This is also evident in the peripheral circulation, where only 3% of cells pre-therapy express high levels of ICOS, but the number goes up five-fold after treatment with anti-CTLA4. ICOS levels are therefore an easy, objective marker to assess the impact of anti-CTLA4 .

ICOS is a closely related homolog of CD28 and CTLA-4. It binds to a molecule called B7h. Its function is controversial: it is known to play a role in tuberculosis and is expressed on regulatory T cells, and there is data suggesting it is important for the survival of Th1 cells.

When the bladder cancer patients are typed for NY-ESO-1, anti-CTLA4 is seen to induce a population of ICOS-high cells that recognize those expressing NY-ESO-1. Those cells also have higher levels of phospho-AKT. ICOS may be creating a survival signal on top of the proliferative signal generated from blocking CTLA-4, essentially producing a more robust anti-tumor effect[4].

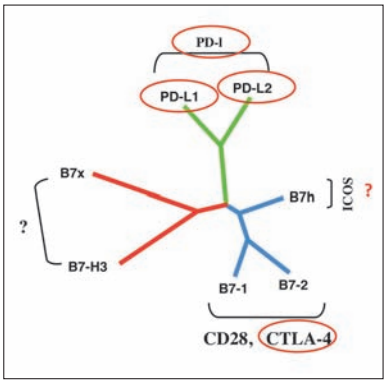

Figure 5
Extended B7-CD28 family.

If responders and non-responders are separated, all seven of the seven responders had a two-fold or a higher increase in ICOS-positive cells. Of those that went on to develop progressive disease, one showed an increase and one did not. Among five patients who died within the assay period, some had high pre-existing levels, but none had more than a two-fold increase of ICOS cells sustained over 12 weeks.

So, there are two factors that predict a good response. One is pre-existing immunity as assessed by antibody titers, and the second is high levels of ICOS sustained at 12 weeks. This sustained elevation of ICOS-high CD4 T cells correlates with survival.

Tracking the molecular responses in individual patients and even individual lesions may help provide more clarity on the nature of the responses.

In one example of a complete responder, a patient with melanoma was given chemo and an adjuvant vaccine, but his disease was progressing. He had a tumor in the left upper lung that was resected, a new tumor in the right lower lung, and one in the inguinal nodes. He had failed chemotherapy with carboplatin+VT and then was given anti-CTLA4.

After being given anti-CTLA4, his levels of FOXP3 went down, indicating that the number of regulatory T cells in the blood had decreased, and the levels of ICOS increased in the blood. There was an increase in both the white blood cell count and the absolute lymphocyte count that went up and stayed up.

Another patient with melanoma who had a toe amputated was also given a vaccine and high-dose IL-2, and after failing chemo, had progressive disease. When this patient was given anti-CTLA4, FOXP3 levels decreased, but so did ICOS levels. There was a transient increase in the absolute lymphocyte count, but a further fall in the white blood cell count. This patient's disease continued to progress and after failing chemotherapy again, the patient died.

Negative regulators

Anti-CTLA4 clearly has a lot of promise, but needs to be better understood and combined with other therapies to improve response. Some experiments show that actively engaging ICOS with agonists can enhance the therapeutic effect of anti-CTLA4. In the CD28 extended family, CTLA-4 is a good checkpoint to block and ICOS is a positive checkpoint that helps tumor response.

Another negative regulator that's on activated T cells is PD-1, which has two ligands. PD-L1 is present only on APCs; PD-L2 is found on many tumor cells. Another branch of this family includes B7x and B7-H3. The ligand for these is unknown, but in vitro data suggests that that they are inhibitory. They are very different from all the others because they're never found on hematopoietic cells. They're found deep in the tissue, but they're also found on tumor cells.

To determine B7x's function, a transgene with the rat insulin promoter is over expressed in the beta cells of a mouse pancreas. These mice are then crossed with BDC2.5 mice, which have a T cell receptor transgene that destroys their beta cells. All of the resulting mice get diabetes.

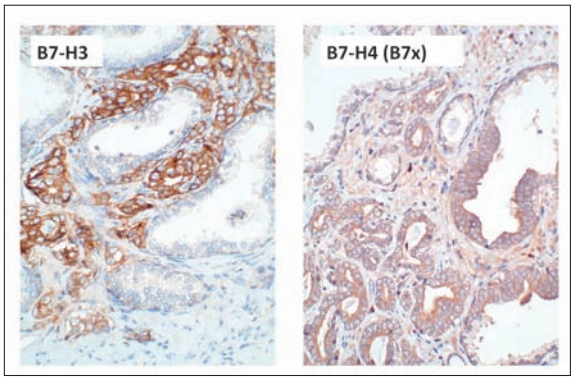

Figure 5
B7x and B7-H3 are both over expressed in human prostate cancer.

But if the B7x molecule is over expressed, the beta cells then become completely resistant to the induction of diabetes, even though every T cell in the body can see those beta cells. This is interesting because both B7x and B7-H3 are over expressed in human prostate cancer.

In an 800-patient retrospective study of tissue arrays, high expression of one or both of B7x and B7-H3 correlates with extracapsular extension, seminal vesicle invasion and non-organ confined disease.

It also corresponds with a risk of biochemical recurrence and, most important, with greater risk of clinical failure and of death from disease following prostatectomy within seven years. These observations suggest that the molecules are certainly harmful, and worth blocking[5].

With all these targets, including CTLA4, PD-1, and B7, this whole field is exploding. The molecules all work on different steps in different pathways.

A couple of years ago, colorectal carcinomas and breast cancers were resequenced to find mutations, which potentially generate new antigens. Putting those sequences through algorithms predicts new epitopes.

For HLA-0201 alone, there are on average nine neo-epitopes per tumor. Assuming six MHC molecules per tumor, there are an estimated 54 total neo-epitopes per tumor generated by genomic instability alone.

Even if the algorithms are wrong 95% of the time because the gene isn't expressed or isn't properly processed, there still would be five or six neo-epitopes per tumor. They may not elicit an immune response because CTLA-4 and these other molecules limit the response, but it may be possible to harness the immune response.

With an effective checkpoint blockade, one drug can be turned into six or seven drugs and potentially generate an effective tumor response, essentially creating a combinatorial approach with a single drug.

These therapies can be described as immunosupportive. Anti-CTLA4, for example, is completely benign. So a lung cancer treatment with antagonist blockers of epidermal growth factor (see Varmus, page 9) could be supplemented with anti-CTLA4, which would let the immune system thrive and take out tumor cells more effectively.

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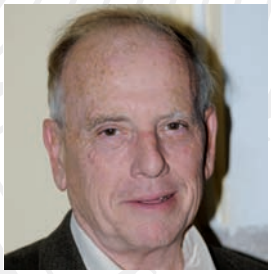

Signal transduction therapy of cancer at a crossroads

A report on a lecture by
Alexander Levitzki
The Hebrew University of Jerusalem, Jerusalem, Israel

Human cancers represent heterogeneous populations of cells whose genomes constantly change, so targeted therapies must be aimed at continuously changing networks. To overcome this difficulty, one promising strategy involves using a ligand-guided vector carrying a double-stranded RNA (dsRNA) such as poly-inosine/cytosine, or polyIC. Upon ligand-induced receptor internalization, polyIC triggers the production of cytokines as well as chemokines, which in turn attract immune cells to the tumor. In mice, a vector carrying polyIC, melittin, polyethyleneimine, polyethyleneglycol and the epidermal growth factor (EGF) eradicates tumors that over express the EGF-receptor using either local or systemic application. The treatment also eliminates mixed tumors, in which about half of the cells over express EGFR and the other half carry a truncated version of the receptor. This 'bystander effect' is mediated by polyIC-induced cytokines generated by the tumor. Immune cells that accumulate in the tumor also produce pro-apoptotic cytokines. Reconstituting the immune system of the SCID mice with human peripheral blood mononuclear cells immediately after treatment keeps the mice alive and cancer-free. ***Alexander Levitzki*** *suggested that this strategy could be utilized to treat any cancer with a significant population of cells that over-express a protein that can be induced to internalize by a ligand.*

The nature of cancer is such that its genome instability leads to constantly changing signaling networks. As a result, targeting only a small number of elements fails to cure the cancer. The drug cocktail needs to aim at a moving target in order to have a real impact on the disease[1].

In the case of multiple myeloma, for instance, the cocktail is modified as the disease progresses, indicating that this might be a valid strategy. The approach has increased the life expectancy of people with multiple myeloma from roughly two or three years to about ten years.

None of the targeted therapies introduced thus far have generally performed as well in the clinic as Gleevec has for early chronic myelogenous leukemia (CML). Some targeted agents prolong life by weeks to months, but often, no extra benefit is seen with these targeted agents as compared with classical chemotherapy.

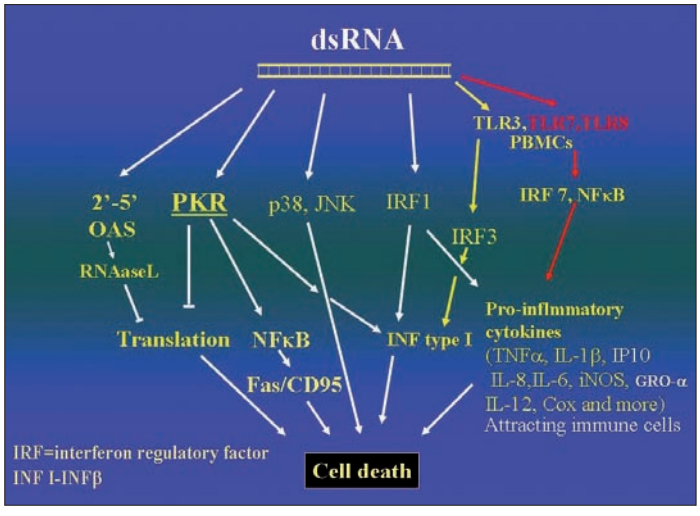

Figure 1
The strategy employs long strand double-stranded RNA of more than 40 base pairs.

There are some benefits to signal transduction therapeutic agents, however. First, they're less toxic than

conventional chemotherapeutic agents. In some cases, as with multiple myeloma, combining these therapies does make a significant difference to survival. Solid tumors are more challenging.

Taming cancer, rather than completely eradicating it, is an expensive and impractical option for most patients. What's needed instead are targeted therapies with a strong 'bystander effect', which allows the killing of tumor cells neighboring the targeted cell. This is important and necessary because, unlike xenografts or transgenic cancer models, the cancer is heterogeneous.

The epidermal growth factor receptor, or EGFR, is a good example for study because it's an important target. EGFR inhibition has only had mixed success so far (see Varmus, page 9). EGFR appears in solid tumors, which are heterogeneous, and express elaborate kinase and other networks. In fact, only a subset of cells respond (temporarily) to the inhibitor, for example.

The concept of 'oncogene addiction' may apply to oncogenic networks but not to single oncogenes, except for BCR-ABL in early CML, which accounts for only a tiny fraction (0.08%) of all human cancers. EGFR is important, but it is not essential.

Still, EGFR remains an attractive target because it is over expressed in many cancers, including glioblastoma, head and neck cancer, breast cancer, non-small cell lung cancer and various epidermoid carcinomas[2].

One useful strategy may therefore be to convert the high number of EGFR into the Achilles' heel of the tumor. For example, it may be possible to activate apoptotic programs selectively in cells that over express EGFR.

Immune recruitment

For historical and scientific reasons, this strategy employs long strand double-stranded RNA of more than 40 base pairs, rather than short interfering RNA to shut down genes. Because this is a household response in almost every cell in the body and orchestrates a number of pro-apoptotic pathways, including the recruitment of the innate immune system to the tumor.

In a cell that over expresses EGFR, the EGFR-targeted dsRNA would trigger cell death as well as a bystander effect, leading to the demise of neighboring tumor cells, induced by the cytokines and the immune cells that converge on the tumor.

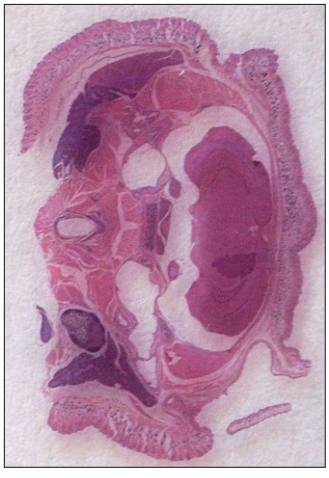

Figure 2
A three-day treatment of the vector loaded with polyIC kills the tumor.

Poly-inosine/cytosine or polyIC is a classic dsRNA that has been used for years. Systemic application of polyIC is unpleasant and toxic, which is why it has never made it to the clinic as a routine treatment. The goal is to use polyIC as a Trojan Horse, internalizing it selectively into cancer cells that over express EGFR.

The vector is built from polyethyleneglycol (PEG) with two active ends. One end is hooked to the amino terminal of EGF, and the other end to polyethyleneimine, which is complexed with polyIC. At the other end of PEI the peptide melittin is bound.

The vector has to be built in a way that allows the EGF to retain its binding activity and allow it to internalize with the receptor. Melittin facilitates the release of the contents into the cytoplasm because it activates phospholipase A2, localized to the membrane of the endocytotic vesicle into which the dsRNA-loaded vector internalizes. The dsRNA goes into the cytoplasm, which is where the signaling occurs.

When the vector is loaded with dsRNA, cells that internalize the polyIC vector die. Isogenic cell lines, which carry a truncated version of EGFR that is common in glioblastoma and other tumors, do not internalize the

vector and do not die. Apoptosis only occurs in cells that over express the wild type EGFR in high numbers. Mellitin enhances the effect by almost a factor of 10, although it is possible to achieve the same effect without melittin, using another form of PEI.

The internalization can be monitored either by looking at EGF using the right antibody or by staining the PEI. Using one of these methods, it's clear that the cells take up the vector. The vector begins to diffuse in the cell after its release, and this process happens faster with mellitin. Without mellitin, the vector remains in the vesicles for a longer time, but is eventually released.

Once the vector is released, cancer cells that over expresses EGFR die. In contrast, cells with few or no EGFR molecules remain alive. All tumors that over express EGFR can be eradicated by such targeted delivery of PolyIC, either by local or systemic application of PolyIC/Mel-PEI-PEG-EGF.

Local delivery

Local application can be used for any tumor model in the animal. For example, in glioblastoma multiforme, which is a fatal disease, about 60% of the tumor over expresses EGFR. Many of these receptors are mutated, but they occur alongside the wild type EGFR within the tumor.

The tumor is implanted stereotactically in the brain, and treatment begins after the tumor has developed. The EGFR-targeted homing vector carrying the dsRNA is delivered stereotactically, by an Alzat pump, as is done in the clinical setting.

If the tumor is grown to about 15 millimeters cubed, the vector alone has no effect. In contrast, a three-day treatment of the vector loaded with polyIC kills the tumor, the mice remain alive for more than a year, and the tumor never returns.

This can be duplicated for all tumors that over express EGFR, including breast cancer, A431 epithelial cancer, head and neck cancer and non-small cell lung cancer.

Using apoptotic staining or EGFR staining a few hours after the treatment begins reveals that the cells that over express EGFR are the ones that die.

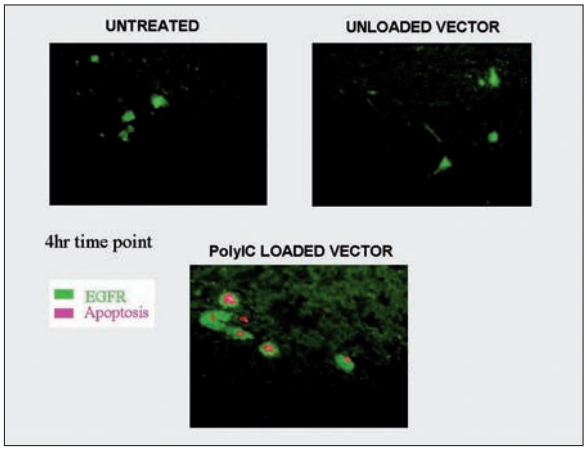

Figure 3
Cells that over express EGFR die by apoptosis.

The treatment works so well because there is a bystander effect. Tumor cells produce interferon-α (IFN-α), IFN–β and other factors induced by the internalized polyIC. These cytokines kill neighboring cells that have not internalized polyIC. Indeed, introducing anti-IFN-α antibodies into the medium inhibits approximately 30% of this bystander killing effect.

The innate immune system is deficient in nude mice used for these experiments, but still plays a decisive role in the *in vivo* bystander effect, as they still harbor NK cells and macrophages.

Tumor cells are generally more sensitive to stress, and succumb to the bystander effect even when they don't express the target. But normal astrocytes and other brain cells are much more robust and they resist killing by the cytokines. This is why toxic effects on normal brain tissue have not been observed.

If the tumor is mixed such that about half the cells express EGFR, and the other half express a truncated version of EGFR — which is not by itself a target for the vector — local application of the treatment will

keep the mice alive because of the bystander effect[3].

The bystander effect is stronger *in vivo* than *in vitro*. If the cells are mixed in a Petri dish, only about 30-40%, and not 100%, of the cells carrying Δ-EGFR (EGFRvIII) die. That's because the bystander effect is stronger *in vivo*, involving immune cells like NK cells and macrophages, that exist in nude mice even if not in large numbers. This is exactly the opposite of small molecules, which work very well in cells, but less potently in whole animals.

Systemic treatment

People don't die from the original tumor that can be excised by radiation or surgery; they die from metastases that in many cases occur much later after treatment. In those cases, the treatment must be by systemic application.

There are protocols in the literature to generate disseminated tumors in the animal. When 1 to 2 million tumor cells are injected into the blood of SCID mice, which are highly deficient in their immune system, they generate tumors everywhere — in the lungs, liver, spleen and so on.

The animals are treated for 3 days on, 1 day off, followed by 4 days on, 1 day off and then another 3 days on. With 15-day (>>200□m) tumors formed from A431 cells, this protocol cures 60% of the animals. With tumors generated from MDA- MB-468 breast cancer cells, the response is excellent, but the mice eventually die.

SCID mice don't have T cells and are deficient in all other constituents of the immune system. But it is possible to reconstitute the immune system and introduce up to 100 million peripheral blood mononuclear cells (PMBCs), which include T cells, NK cells and macrophages, into one mouse.

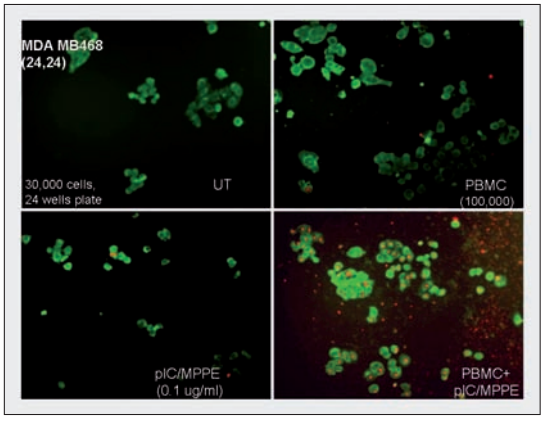

Figure 4
PBMCs and polyIC synergistically kill cells that over express EGFR.

In vitro, treating either MDA-MB-468 breast cancer cells or A431 cells with 100,000 PBMCs or with very low amounts of the polyIC-bound vector generates only a little bit of apoptosis. But when the two are combined, there is massive apoptosis, indicating a high synergy between PBMCs and the polyIC in killing cells that over express EGFR. Cells that do not express EGFR are not killed.

Another type of experiment shows that the medium of the polyIC-treated cells activates PBMCs and that the medium of these treated PBMCs induces a much stronger tumor cell killing as compared with the killing effect of the medium of the original polyIC treated cells.

Regardless of which tumor cells are used as indicator cells, it results in this type of relationship between the parent mediums and the PBMC-treated medium.

In animal experiments, the 10-day treatment with no PBMC is shortened to a 4-day treatment with PBMC, with much superior results. When PBMC is included in the treatment all animals survive and the tumor never comes back[4]. This is not the case when PBMC is omitted from the treatment; the cure rate is much lower.

The reason for this, once again, is the bystander effect. There are cytokines present in the tumor, but not outside the tumor, in the blood. Some cytokines, such as Gro-α, are produced by the tumor even in the absence

of treatment. In the tumor area, the converging immune cells, and not the tumor, produce IFN-γ, IL-2 and TNF-α.

Staining with different antibodies to immune cell components shows that immune cells are converging to the tumor area in the animal. In tumors that don't over express EGFR, PBMCs do not converge on the tumor.

The bystander effect essentially achieves killing in part by the cytokines generated at the tumor, by the internalized polyIC and by the immune cells converging on to the tumor. This strategy may be applicable to many tumors. If tumors have a population of cells expressing a particular protein that can be internalized by a ligand, the ligand can be attached to the vector. The approach is likely to be successful particularly for cancers that over express certain receptors such as EGFR.

The advantages of this approach are that the immune system is targeted to the tumor, and the bystander effect eliminates cells that don't contain the target — in effect, solving the problem of tumor heterogeneity as an impediment to successful treatment.

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In situ immune reactions and tumor resistance: potential therapeutic targets

A report on a lecture by
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The nature of immune reactions that may control tumor invasion and metastasis is of seminal importance for identifying novel targets for cancer treatment. The presence of high adaptive immune infiltration in the center and in the invasive margin of colorectal tumors is the strongest prognosticator of recurrence and disease-free survival, above factors such as tumor stage, differentiation and lymph node involvement. The expression of certain chemokines is associated with the recruitment of selective T cell populations. Modifying the concentration and activity of these chemokines may dramatically change the immune context of a tumor and, subsequently, the patient's clinical outcome. The Toll-Like Receptors TLR7 and TLR8 are expressed in tumor cells, and stimulation by TLR agonists leads to the up regulation of BCL-2 expression, increased tumor cell survival and chemoresistance. Transcriptional analysis of primary lung tumor cells and TLR7- or 8-stimulated lung tumor cell lines reveals a gene expression signature that suggests a TLR chronic stimulation of tumor cells in situ. ***Wolf-Hervé Fridman*** *warned that these data have to be taken into consideration when administering TLR agonists as vaccine adjuvants to treat cancer.*

In view of the poor successes of immunotherapy until recently, it is important to try to understand the interplay between tumor cells and the immune system, and how a natural, unmanipulated immune system can control the clinical outcome of cancer. Pinpointing what shapes this immune system may also help provide tools and targets for designing immunotherapy.

The primary cancer for study in this case is colorectal cancer, which affects 1 million people worldwide. The cancer is often progressive, with only a 40% survival at five years.

Immune cells that infiltrate tumors have been underestimated for quite some time. There are infiltrating cells in the center of the tumor, in the invasive margin and in the lymphoid islets at a distance from the tumor. These are all really important for creating an immune reaction.

The infiltration pattern is different in those patients who have early signs of metastatic invasion, dubbed VELIPI — for venous emboli in the tumor, lymphatic invasion, and perineural infiltration — compared with those who don't.

This observation is based on analyzing a series of 959 colorectal cancer patients, followed for more than15 years, for parameters of immune and inflammatory reactions. The analysis used various techniques, including immunohistochemistry, quantification of gene expression, immunofluorescence of cells and tissue array of selected molecules.

The primary difference in the immune infiltrate between VELIPI+ and VELIPI- patients is the presence of memory cells, particularly memory T cells. Patients with high numbers of memory T cells have no signs of early invasion, and they show good disease-free and overall survival. Patients who don't have these memory T cells show signs of metastasis and have a bad prognosis[1].

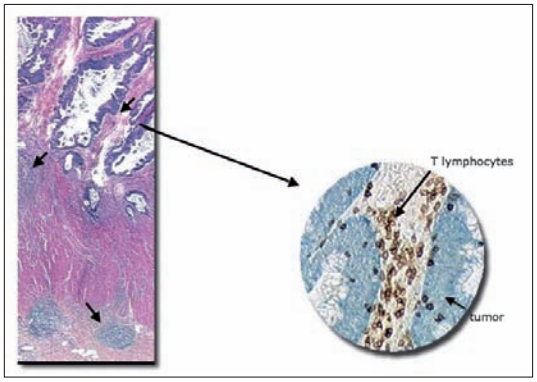

Figure 1
Tumors are infiltrated by T cells.

T cells in the center of the tumor and those in the invasive margin are both important. For example, if there is a high number of memory T cells both in the center and in the invasive margin, the prognosis, overall survival and disease-free survival are excellent, reaching 60 months of survival compared with 12 months for those with low numbers of memory T cells in both regions. This observation applies to memory CD45RO cells, CD8 cells and to granzyme B expressing cells.

The fact that having cells in both the center and in the invasive margin is important indicates that the immune response might need to be coordinated. In heterogeneous situations, in which there are high numbers of T cells in the center and low numbers in the margin, or vice versa, the prognosis is intermediate, albeit more on the lower side. That suggests that it is not just the total number of T cells that is important, but also the location of the cells[2].

Measuring survival

When the 1,000 colorectal patients are stratified by the classical structure of Duke's or TNM, even small non-invasive tumors, which normally are treated with only surgery, have a relapse rate between 20-30%.

If these patients are instead stratified in terms of only two immune parameters, CD3 and CD45RO memory T cells, patients with high infiltrates of these cells in the center of the tumor and invasive margin — including those with lymph node involvement — all have very good prognosis. Even metastatic patients do better.

Intriguingly, patients with low numbers of these cells in the center and in the invasive margin, including those who have very small tumors, behave like metastatic patients and relapse rapidly. These patients are usually only surgically treated, but they obviously deserve other therapies.

In fact, in a multivariate cox analysis, the immune pattern is the only one that stands significantly over the T-stage, N-stage differentiation pattern for disease-free and overall survival for all stage I, II and III patients.

The results suggest that the adaptive immune reaction at the tumor site is important for preventing tumor recurrence. It doesn't eradicate the tumor by itself or make the tumor regress, but certainly plays a role in controlling metastatic cells. The beneficial effect of the adaptive immunity may persist throughout tumor progression, up to stage III, which opens a therapeutic window.

These data provide strong evidence of the importance of the natural anti-tumor adaptive immunity in human cancer. Other groups have reproduced the results in ovarian cancer, head and neck cancer, lung carcinoma and melanoma. It is now more or less an established concept that a proper immune reaction at the tumor site controls tumors.

Analyzing 75 tumors for gene expression, the genes fall by unsupervised clustering into three categories: inflammation, adaptive immunity and immune suppression. Of these, only the adaptive immunity cluster is associated with the control of relapse. Patients with high expression of these genes have only a 20% chance of relapse over 15 years of follow up.

In contrast, people with low expression of these genes have an 80% chance of relapse. There is a gradient for this cluster: the lower the expression of these genes, the stronger the risk of relapse. This cluster of genes on its own appears to control metastasis and relapse.

Coordinated response

If patients are separated by the number of memory T cells in the tumor, genes expressed in memory T cells are obviously highly expressed in those with high memory T cell infiltration, and under expressed in those with low numbers of memory T cells.

Those with high numbers of memory T cells also express Th1 genes, which control CD8 T cell cytotoxicity, in a coordinated fashion. By contrast, genes involved in Th2 responses, which control allergic antibody reaction, show no coordination, nor is there coordination of angiogenesis genes, inflammation genes or immune suppression genes[3].

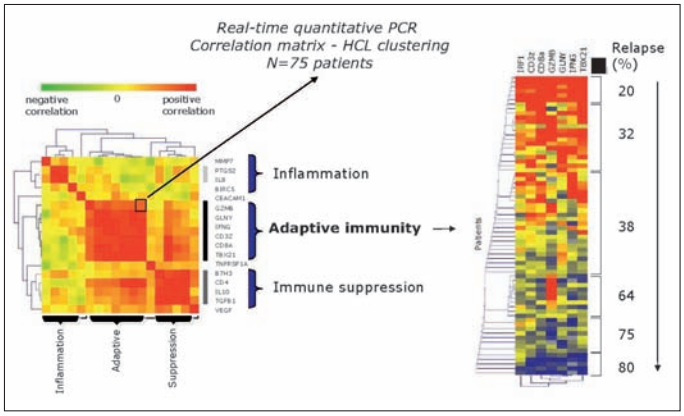

Figure 2
A coordinated adaptive immune response correlates with relapse.

Among patients with a high infiltration of these memory T cells, about 15% do have metastases. The total number of memory T cells is the same between this subgroup of patients and those that are metastasis-free.

But there is one striking difference: in patients with metastases, there are no effector memory cells, even when they have a strong memory T cell infiltrate in general. The coordination of response is therefore important to bring this category of T cells into a state where it can control metastasis[4].

The optimal situation is one in which there is a high infiltration of T cells, with strong immune coordination, large numbers of memory T cells, a cytotoxic Th1 immune response, and low VEGF.

This leads to the production of effector memory T cells, which don't so much control the primary tumor, but prevent metastatic cells from disseminating, and perhaps control memory T cells.

This group of patients has only a 10% rate of metastases over 10 years. But high levels of VEGF induce neovascularization. Even in the presence of T cells, tumor cells vascularize, and the rate of metastases rises to 50%. When the T cell infiltrate is lost, there is no immune coordination, and 80% of the patients develop metastasis.

Attractive environment

Some of the elements that shape this efficient immune response are ICOS (see Allison, page 45), STAT-1, TNF and the PD ligand family. By genomic neighborhood sequence homology, co-occurrence and phylogeny, gene expression, and literature co-occurrence, an *in silico* search reveals other genes associated with these elements.

The number one hit is CX3CL1, a chemokine gene that attracts hematopoietic cells and other chemokines. The others are CXCL9, CXCL10, CXCL2, interferon-γ receptor, MADCAM1 and ICAM — essentially, a series of genes that are involved in adhesion, in recruiting cells, and in shaping and modifying the infiltrate of inflammatory cells.

These predicted genes are associated with the disease-free survival of the patient. High levels of CX3CL1, CXCL9, CXCL10 and MADCAM1 are associated with disease-free survival, and low levels with a bad prognosis. In contrast, the chemokine CX3CL5 is not predicted, and its levels have no effect on survival.

If the levels of an adhesion molecule are combined with those of a chemokine, the predictive value becomes even more accurate.

Expression of the genes for CX3CL1, CXCL9 and CXCL10 is associated with a very high lymphocytic infiltrate. When these genes are not expressed, or are expressed at very low levels, there are no infiltrating lymphocytes, suggesting that these products bring the lymphocytes into the tumor.

The chemokines don't all attract the same cells. CX3CL1 seems to attract effector T cells and Th1 cells, but not memory T cells. In contrast, CXCL9 attracts memory cells, but not cytotoxic T cells or Th1 cells.

Correlation matrix analysis reveals that these chemokines do cluster with different populations. CXCL9 and CXCL10 cluster with memory cells and CX3CL1 clusters with CD4 and CD8 cells, total T cells and NKT cells. Other chemokines outside of the cluster also attract NK cells and B cells, but for a coordinated immune response, the tumor must have high expression of at least these three cytokines.

Immune interface

Lung cancer is the most common cause of cancer death in the world. The lung is interesting particularly because it is the interface between the body and the external environment, and it experiences a lot of viral infection, bacterial infection and tobacco-related inflammation.

In more than 200 lung carcinomas — two-third adenocarcinoma, one-third squamous cell carcinoma — at an early disease stage with none or low lymph node infiltration, the data recapitulate the results from colon carcinoma.

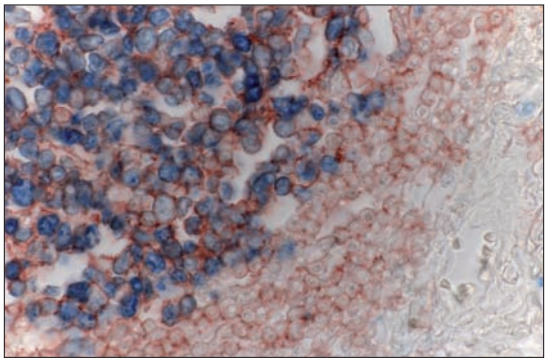

Figure 3
Presence of tertiary lymphoid structures in early-stage non small cell lung cancer.

Among the infiltrating hematopoeitic cells, most are T cells, and there are some B cells and NK cells. The T cells in the infiltrate have a bias toward CD8 cells as compared to those in lymph node or blood. They are mostly of the memory phenotype, whereas there are mostly naïve T cells in the blood, and of equal numbers in the lymph nodes.

B cells in the blood are mostly of the naïve phenotype, the lymph node has cells with a memory phenotype, but no plasma cells, and the tumor mostly harbors memory cells and plasma cells.

The tumor, both in colorectal and lung carcinomas, has memory T cells, CD8 cytotoxic cells and memory B cells. In the vicinity of the tumor, there are tertiary lymphoid structures, which look like germinal centers with a T-cell zone and a B-cell zone.

The T-cell zone is the only site with mature dendritic cells, the professional antigen-presenting cells, and the B-cell zone is full of follicular dendritic cells, and also has proliferating, mature B cells — essentially everything needed for an immune response.

This is probably the first place in which the tumor is recognized, and where the immune response takes place. As in the colon, when there is a proper pattern here of mature dendritic cells and mature T memory cells, the clinical outcome is excellent[5].

However, disease-specific survival, which is at 95% after four years, drops to 44% when these tertiary lymphoid structures are lost. The important point is that the prognostic strength of the immune system locally remains very strong in the lung. Other groups have reproduced this for other cancers.

The immune pattern — high or low density of mature dendritic cells, memory T and B cells, Th1 and cytotoxic T lymphocytes—is independent of age, gender, even of the smoking history, tumor histological type and tumor stage and differentiation. High numbers of mature dendritic cells correlate with high numbers of T effector and memory cells and B memory cells, and are associated with a favorable prognosis.

The lung is a site of frequent infection and inflammation. Chronic inflammation can promote the development and progression of tumors whereas acute inflammation can induce tumor regression. Toll-like receptors (TLRs) are key molecules in the response to infections and inflammatory processes.

TLRs are analyzed in lung tumors, with particular interest to TLR7 and TLR8. These TLRs are intracellular receptors that recognize double-stranded RNA viruses, and are expressed on the cells of the immune system.

TLR7 and TLR8 are expressed in lung tertiary lymphoid structures, and there is more TLR7 than TLR8 within those germinal center-like reactions. They are not expressed in normal alveolar cells, but are highly expressed in bronchial cells, from which some or most of the tumor cells originate.

Managing inflammation

The expression of TLR7 in tumor cells is sometimes high and sometimes low, but TLR8 is always expressed at high levels. Well-known cell lines of adenocarcinoma and squamous cell carcinoma express all TLRs and particularly TLR7 and TLR8 proteins.

Stimulating the A459 cancer cell line with a TLR7 agonist, a TLR8 agonist, or an agonist of both, increases the number of cells after 10 days, although this doesn't seem to be induced by increased proliferation. The cells seem to show some resistance to apoptosis, because there is increased expression of BCL-2.

The cells not only have a better survival, but an interesting and somewhat cumbersome finding is a reduced resistance to chemotherapy. In a colony assay of a squamous cell carcinoma, pre-treating cells with a TLR7 agonist completely protects the cells from destruction with chemotherapeutic drugs. Pre-treating with a TLR4 agonist has some effect, although it is much less pronounced[6].

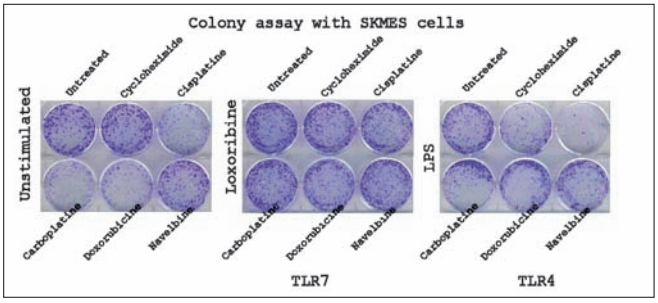

Figure 4
TLR7 stimulation induces chemoresistance to platinum salts particularly.

In conclusion, the stimulation of lung cancer cell lines induces survival of lung cancer cell lines, resistance to chemotherapy-induced cell death and modulation of gene transcription. It also induces up regulation of CCR4 and down regulation of FN1 — which may make these cells more likely to better metastasize — up regulation of BCL-2, and a gene signature of activated non-small cell lung cancer cells.

At a time when people are using TLR7 and TLR8 agonists in the treatment of melanoma or basal cell carcinoma, or as an adjuvant with vaccines, it is worth noting that TLR7 and TLR8 stimulation may be a double-edged sword.

Both TLR7 and TLR8 are expressed on immune cells, and their stimulation is beneficial. The immune cells will better survive, better proliferate, better differentiate and better kill and control the tumor.

But when they are expressed on tumor cells — and this should be tested before any treatment with the agonist on the tumor cells — they will also induce better survival and better proliferation of tumor cells, and perhaps also resistance to chemotherapy.

The general conclusion is that in colorectal and in non-small cell lung cancers, local chemokine production may shape an efficient adaptative immune microenvironment and control tumor metastasis, recurrence and patient survival by bringing a a selected set of T cells into the tumor.

Infections and inflammation may induce TLR7 and TLR8 expression on tumor cells, favoring resistance to apoptosis and chemoresistance. This balance should be taken into account when patients are treated, and in the development of new immunotherapeutic drugs targeting adapative or innate cell molecules and receptors.

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PART IV: Signaling pathways

Therapeutic targeting of the PI3K pathway in cancer

A report on a lecture by
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Novartis Institute for Biomedical Research, Cambridge, USA

The comprehensive annotation of human cancers at the molecular level is becoming evermore a reality, enabled by a number of large-scale public efforts. Available cancer databases have amassed detailed molecular data for a wide variety of cancers. Developing pre-clinical hypotheses for directing the rational testing of therapeutics in phase I trials will require profiling of highly annotated cancer models that are themselves highly annotated and thus "connected" to authentic human tumors. To enable this goal, there are efforts under way to build a cell-line encyclopedia, a repository of 1,000 cancer cell lines, and a high-throughput system that would allow these models to be interrogated with certain perturbations. Pooled shRNAs also have the advantage of overcoming the throughput issue because thousands of shRNAs can be analyzed in a single experiment. Looking at dozens of cell lines at once, instead of analyzing one cell at a time, dramatically enhances the signal-to-noise ratio. ***William Sellers*** *described examples in which information about cell lines at a genetic or molecular level has helped make robust predictions of clinical response. Genetic-based deregulation of PI3K signaling is among the most common somatic alterations in human cancer. Sellers also described BEZ235, a dual mTOR/PI3K inhibitor in phase I clinical development. Intriguingly, a RAS-driven lung cancer model appears refractory to BEZ235 as a single agent, but is sensitive to combinations of BEZ235 and a MEK inhibitor underscoring the growing need to understand therapeutics both as single agents and in combination.*

For most human cancers, the tumor is first analyzed at a genetic or molecular level and that information is then connected to therapeutics. But that approach is only useful when there are models available that reflect the true nature of the cancers being treated.

There are efforts under way to build such models, and to try to relate the models to human cancers by understanding their molecular constituents. The molecular data for most cancers is available in public databases, and efforts such as the cancer genome project will add to those databases.

The NCI-60 is a set of 59 human cancer cell lines derived from diverse tissues. It includes gene expression data, comparative genomic hybridization data using a 100K SNP array, and mutations identified with Sequenom genotyping. Orthogonal analysis of these data taken together could lead to either biological or therapeutic insights.

In parallel, the NCI-60 can also be used to interrogate drug sensitivity against genetics, and ask whether there are intersecting data that would provide a hypothesis that's testable in the clinic.

For example, NCI-60 data is separated into BRAF-mutant versus non-BRAF mutant[1]. In this case, the non-BRAF mutants also include RAS mutations. Instead of interrogating expression data, the IC50 data that has been developed by the NCI-60 for 70,000 compounds is analyzed.

For the set of classifiers that include the BRAF mutants compared with wild type, there is a strong

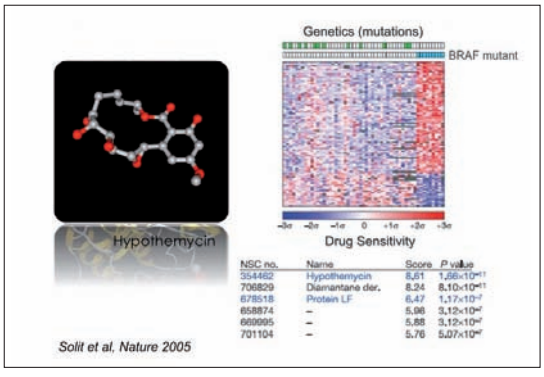

Figure 1
Interrogating BRAF mutations and drug sensitivity.

set of compounds that are highly statistically enriched for their lower IC50. Among those compounds is hypothemycin and Protein LF. Hypothemycin is both a MEK and ERK inhibitor and has been co-crystallized with the ERK kinase domain, suggesting that MEK inhibition in the context of BRAF mutation might be particularly effective. Others have also shown in animal models that SKMEL28 cells, which are BRAF-mutant melanomas, are sensitive to the Pfizer MEK inhibitor PD325901. This compound is a selective MEK inhibitor, compared with hypothemycin, which is relatively non-selective.

This is an example in which a pre-clinical hypothesis was generated, and the data suggests that the approach might work in the clinic. Information about the cell lines at a genetic or molecular level is able to help make more robust predictions for what would happen in the clinic.

Expanded models

Despite the strong statistical findings with respect to BRAF mutation, the inherent problem is that the NCI-60 dataset is too small. For example, there are only four cell lines with mutations in phosphoinositide-3 kinase (PI3K), so it is difficult to generate statistically significant data. The number of model systems must be expanded and a comprehensive system is needed for interrogating those models with certain perturbations.

The Broad institute, Novartis and the Genomics Institute of the Novartis Foundation in La Jolla have together established a collaboration to develop the cell line encyclopedia project, a repository of about 1,000 cancer cell lines.

To ensure that the lines have all been fully analyzed and don't differ in different sites, the 1,000 cell lines are being collected anew, grown in central locations, and mirror repositories created at all three sites, as well as the three Novartis oncology sites.

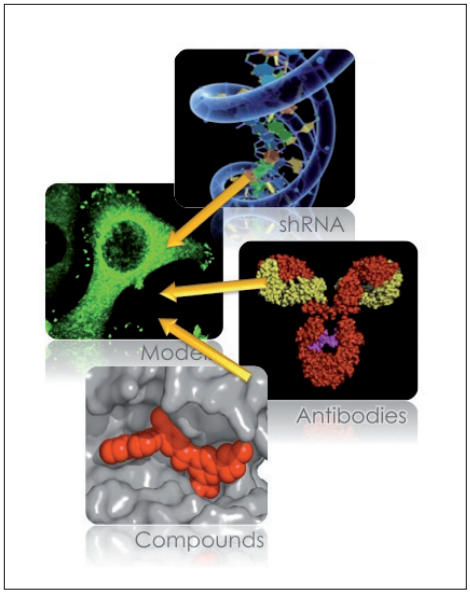

Figure 2
Connecting patient-disease-therapy.

All of these models will be fully annotated for mRNA, DNA copy number alterations, LOH and sequence data by Solexa for 1400-1500 genes. A number of cell pellets have also been frozen, in case new proteomic technologies or metabolic analyses become available. Data from these cell lines will be publicly released.

A new high-throughput system is being built in order to interrogate these cell line models. In the meantime, in collaboration with Jeff Settleman, a small group of Novartis compounds is also being tested against a collection of up to 600 cell lines at Massachusetts General Hospital, showing the relative high, medium and low sensitivity distribution of the cell lines.

So far, the project has 630 cell lines that have been grown and expanded in all the mirror sites. High-density SNP arrays have already been used to generate LOH and copy number alteration data for the first 243 cell lines.

Still, cell lines are not the perfect way to model cancer, as they often don't reflect the true genetic or molecular characteristics of tumors. For instance, cell lines grown in plastic culture dishes may not accurately recapitulate developmental pathways such as the Wnt, Notch and Hedgehog signaling pathways. So, in a parallel effort, primary tumors are being grown in nude mice and monitored for tumor growth.

At passage 1, the tumors are fixed and frozen and the histology studied. At passage 2, there are generally enough tumor fragments available to conduct a larger scale efficacy study. Both primary tumors and passage 1 and 2 tumors are available for SNP arrays and expression profiling.

So far, more than 500 primary tumors have been brought in. Of these, about 140 are growing in mice, and there are about 80 primary tumor models in which it is possible to conduct drug trials.

One of the limitations of this approach is that there is not enough throughput to analyze one drug across 80 tumors. There needs to be some technological advance to allow for a higher capacity analysis of tumors implanted in mice.

Still, this is an interesting way to get to the next step of tumor models. The histology of the patient tissue, for example, looks similar to that of the xenograft, which is not usually the case for most cell lines.

Functional interrogation

Pooled shRNA has the advantage of overcoming the throughput issue because thousands of shRNAs can be analyzed in a single experiment. To begin to develop pools of highly annotated shRNAs, an automated shRNA validation platform measures the ability of every Broad TRC1 shRNA vector to knock down endogenous mRNA in two independent cell lines analyzed by Q-PCR.

This is done at fairly low MOI of 20% to ensure single-copy infection, and the validation is in two cell lines having a greater than 70% mRNA knockdown. In general, at least in the first generation libraries, more than 50% of the shRNA constructs are inactive. From a signal-to-noise ratio, that's not a good starting position underscoring the importance of moving to validated libraries.

After completing shRNA validation for several thousand genes, druggable targets are the first priority. In one example, multiple cell lines have been used to look at synthetic lethality in the context of BRAF. Initial attempts to use shRNA have been limited by the small number of cell lines used. In this case, shRNAs were taken in a well-by-well approach and the suppression of cell growth analyzed in 28 cell lines.

Of the 28 lines, there are 5 BRAF mutant cell lines: 2 colon cancers, 2 melanomas, and a breast cancer cell line. Interestingly, there are four V600E mutations. One of them, the A2058 melanoma cell line, has a PTEN deletion. There is the MDA-MB-231 breast cancer line with an unusual BRAF mutation, G464V. This cell line also has a KRAS mutation, one of the few to have both a RAS and a RAF mutation.

Depending on which of these mutant BRAF cell lines is used, the answer is different. Only in one of these, the HT-29 colon cancer cell line, does BRAF score as the number one shRNA, and only in three cell lines is BRAF considered sensitive based on a Z score. In the other two cell lines, one of which is PTEN-null, BRAF doesn't even score.

However, instead of looking at the data one cell line at a time, if the 28 cell lines are divided into classes (BRAF mutant vs wild type) and analyzed together, BRAF is the number one hit, scoring as preferentially growth inhibitory to BRAF mutant cell lines. Second on the list is MEK, which didn't even show up in the other analysis. Using this approach overcomes the noise from each individual cell line, and is instructive of the need for larger data sets[1].

Understanding drug action

The inhibitor of apoptosis (IAP) program uses shRNA profiling as a way to generate patient-stratification insights. The IAP program began with the idea that XIAP, the X-chromosome linked inhibitor of apoptosis, functions by sequestering caspase-9.

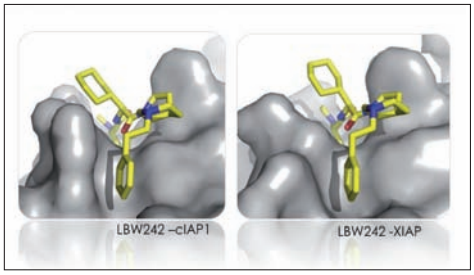

Figure 3
Targeting the inhibitors of apoptosis or IAPs.

Mitochondrial damage releases cytochrome c and SMAC, the second mitochondrial activator of caspase. SMAC displaces caspase 9 from XIAP and triggers apoptosis. The original hypothesis was that the intrinsic pathway of apoptosis is regulated by a rheostat, established at least in part by the abundance of IAPs in the cell.

Drug discovery efforts in this area have been informed by the finding that the BIR3 domain of XIAP can bind to SMAC. The minimal domain of SMAC required to bind XIAP is a four amino acid sequence at the N-terminal. From a protein-protein interaction perspective, this is a small interaction surface, and led to the idea of creating so-called SMAC mimetic small molecules.

A de-peptidization strategy helped convert this highly active *in vitro* peptide to a small molecule that can also work in animals. However, LBW242, a SMAC mimetic, binds to XIAP, but also to CIAP1 and presumably CIAP2. It is not a selective XIAP inhibitor and can have multiple functions, based on the function of XIAP, CIAP1 or CIAP2 in the cells.

LBW242 does not induce normal cells to die by apoptosis and in a panel of 509 cancer cell lines, for example, only 3% are sensitive to the drug at a concentration lower than 1 micromolar, 10% at a concentration less than 10 micromolar, and the rest are quite insensitive.

This means there's something very specific about the cell lines that do respond to LBW242. The cell lines span a range of tumor types, including renal, pancreatic and breast cancers, so there is no tissue-specific rationale for their sensitivity. Gene expression signatures for the sensitive cell lines have also been uninformative.

There may instead be something about the dependence of those cell lines on downstream components in the pathway, specifically the nature of caspases or IAPs, that can predict sensitivity.

The first prediction is that these cells should die in a caspase 9-dependent manner. This was the entire basis of the drug discovery hypothesis. However, an siRNA to caspase-9 has no effect on the IC50 in these cell lines. Instead of dying in a caspase 9-dependent manner, these cell lines are dying in a caspase 8-dependent manner.

That changes the entire hypothesis from one in which the presumed mechanism of cell death is through the intrinsic apoptotic pathway to one in which the extrinsic apoptotic pathway is somehow involved.

Supressing lethality

Lethality suppression, in yeast genetic terminology, is the result of a genetic event that has a phenotype of lethality which, when combined with a second genetic event that either has no phenotype or has a positive phenotype, leads to a viable outcome. Thus the second genetic event suppresses the lethality induced by the first genetic event.

Lethality suppression can also be used to screen drugs: a drug is like a genetic event giving a lethal phenotype, and then either an RNAi or, for example, a second drug has either no phenotype or a positive growth phenotype. The first drug combined with the RNAi results in viability.

This approach has one major advantage: positive selection under a lethality suppression screen generates a very high signal-to-noise ratio. The number of false positives that result from promoting growth should theoretically be much lower than in growth suppression.

In a lethality suppression screen with plated SKOV-3 ovarian cell lines, cells are transfected with the apoptome

siRNA library in a well-by-well format. Most cells are viable and unaffected by the DMSO control, but in the presence of LBW242, the cells become inviable. There are a number of siRNAs in specific wells that rescue viability, including tumor necrosis factor (TNF)-α, TNF receptor 1 (TNFR1), RIPK1 and other members of the apoptotic cascade[2].

Surprisingly, XIAP, the drug target in the original hypothesis, is itself a rescuer. This suggests that XIAP cannot be the dominant target in these cells, and that extrinsic activation of the TNFR pathway is critical for cell death mediated by LBW242.

Adding the drug to three sensitive cell lines results in at least a 10-fold induction of TNF-α and in cell death; insensitive cell lines don't show this response. A soluble TRAIL receptor cannot block cell death in the sensitive cell lines, whereas soluble TNFR successfully blocks cell death, suggesting that the induction of TNF-α is required for cell death mediated by LBW242.

The levels of XIAP are noticeably unchanged, but at increasing concentrations of the drug, CIAP1 is degraded. This is presumably linked to the RING domain and to the ubiquitin ligase function of CIAP.

In this model, CIAP-1 regulates NIK downstream of TNF-1, typically degrading NIK. However in the context of a SMAC mimetic molecule, CIAP-1 is degraded and NIK is stabilized, activating non-canonical and canonical NF-κB pathways, with the induction of TNF-α as a mediator of cell death.

Those functional data — as opposed to a gene expression signature — lead to a very specific hypothesis: patients whose tumors are high for TNF might be more sensitive to the inhibitor.

Screening with Affymetrix expression arrays shows that of 33 available xenografts, a non-small cell lung cancer xenograft has the highest elevated basal TNF-α mRNA expression. This xenograft is fairly sensitive to LCL161, the clinical candidate, and to LBW242.

This doesn't prove that TNF is the key, but provides a hypothesis to survey patients in the clinic for TNF levels and try to correlate those levels with efficacy. The second thing it does is to provide a biomarker, which is the degradation of CIAP-1.

This same type of shRNA screen is being done with pooled shRNAs as the first proof-of-concept. The 2,089 shRNAs from the apoptome are used to create a normalized DNA pool. The DNA are used to transfect 293 cells, a lentiviral pool is produced and used to transfect control cells, and then the distribution of the shRNA pool at its baseline is assessed by Solexa sequencing. When the data is not normalized, there is a five-fold range between the highest and the lowest shRNAs in the distribution.

The packaged lentivirus is also used to infect SKOV-3 cells that are sensitive to LBW242, which are then selected in puromycin for thee days. The experiment is repeated by treating the cells with DMSO or 5 micromolar of the IAP inhibitor, waiting for 7 or 14 days, and the DNA is then isolated. Solexa sequencing deconvolutes the proportion of shRNAs that are present at the beginning of the experiment and in each of the treated or untreated pools at day 7 and day 14.

The data recapitulate a number of the observations that were made initially with TNFα-R1, TNFα-R2, XIAP and caspase 3 that were present in this pool.

Synthetic lethality

One of the proofs of synthetic lethality in the clinic is from a key mutation in the PI3K pathway: the genetic inactivation of TSC1 and TSC2 in the disease tuberous sclerosis (TSC). The disease is characterized by angiomyolipomas, which are mostly renal benign tumors, cortical tumors or the subependymal giant astrocytomas (SEGA), and seizures.

An mTOR inhibitor known as RAD001 has been in pediatric clinical trials in TSC for both angiomyolipomas and SEGAs. Pre- and post-treatment at 5 mg/day, there is a dramatic reduction in the size of the tumors. But more importantly, every patient in the trial has responded by RECIST criteria. Unexpectedly, seizure control is also improved.

These exciting results suggest that understanding the role of a tumor suppressor gene and, at least in one case, the immediate downstream activating consequences, can help make progress in disrupting the pathways of tumor suppressors.

BEZ235 is a dual inhibitor of mTOR and PI3K[3]. The diverse mechanisms for activating PI3K can lead to difficulty in formulating specific narrow clinical hypotheses. For example, PI3K inhibitors might work in EGFR-driven tumors, HER2-driven tumors, p110α-mutant tumors and PTEN-deletion tumors. They might also be anti-angiogenic. This may lead to a desire to test PI3K very broadly, yet it's still likely preferable to test a selective set of hypotheses, particularly to develop effective combinations that target specific populations.

For example, in the Tet-op-PIK3CA H1047R–CCSP-rtTA double-transgenic mice, adminstration of tetracycline results in tumors driven by the specific mutation in the lung, and this can be followed by treatment with BEZ235. PET and CT scans reveal that the tumor completely regresses.

In a phase I trial of this inhibitor, 3 of 7 patients who harbor mutations in either had a prolonged course on therapy without progression, whereas none of the patients with wild type status responded. These data are quite preliminary, but are trending in the right direction[4].

Finally, the LSL K-RAS allele is used to address whether RAS activation confers selective dependence on PI3K. In this case, the BEZ235 inhibitor has no effect on the RAS-dependent tumors. However, when used in combination with a MEK inhibitor, BEZ235 dramatically reduces the tumor size. The exploration of combinations of dual pathway inhibitors will be an important consideration for clinical development in the future.

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Discovery of a new class of mTOR inhibitors reveals rapamycin-resistant outputs of mTORC1 and mTORC2

A report on a lecture by
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The mammalian target of rapamycin (mTOR) regulates cell growth and survival by integrating nutrient and hormonal signals. These signaling functions are distributed between at least two distinct mTOR protein complexes, mTORC1 and mTORC2. mTORC1, but not mTORC2, is sensitive to the selective inhibitor rapamycin and activated by growth factor stimulation via the canonical PI3K/AKT/mTOR pathway. Growth factor stimulation of PI3K causes activation of AKT by phosphorylation at two key sites, T308 and S473. The central role of mTOR in controlling key cellular growth and survival pathways has sparked interest in discovering inhibitors that bind to its ATP site and therefore target both mTORC2 and mTORC1. Two novel and specific mTOR kinase domain inhibitors, PP242 and PP30, are the first specific active-site inhibitors of mTOR and therefore the first specific inhibitors of mTORC2; they block phosphorylation of AKT at S473 and prevent its full activation. These compounds also inhibit proliferation of primary cells more completely than does rapamycin. ***Kevan Shokat*** *reported that although PP242 inhibits both mTORC1 and mTORC2, its enhanced ability to block mTOR signaling is based on its more effective mTORC1 inhibition than that of rapamycin, and that its activity against mTORC2 is not the basis for this enhanced activity.*

There is great interest in developing lipid and protein kinase inhibitors to treat a number of diseases. In cancer, for instance, targets that are either amplified or hyper-activated can lead to an increase in the proliferation or growth of cancer cells. Designing selective inhibitors of those oncogenically activated kinases may be able to shrink these tumors and treat them.

Cancer cells have an amazing ability to adapt. They can, for instance, display oncogenic switching from the epidermal growth factor receptor to MET when treated with specific inhibitors. There are also examples of resistance intrinsic to the kinase domain so that mutations in the gatekeeper don't allow the small molecule to bind.

What's even more impressive is that the pathway itself is adaptable, without resorting to oncogenic switching or point mutations. The pathway is able to adapt to the presence of the first small molecule inhibitor and up regulate another of its components in order to gain back proliferation.

In some ways, this adaptability is not surprising because one of the hallmarks of signal transduction is adaptation. Cancer cells use this adaptability to thwart small molecule inhibitors. The long-term goal, therefore, is to identify the prime oncogenic kinase, but also to find molecules that hit the nodes of the pathway in a way that short-circuits the feedback.

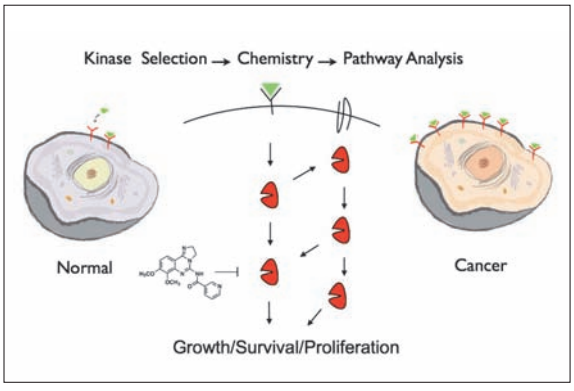

Figure 1
Selective inhibitors of oncogenically activated kinases may be able to treat tumors.

The growth factor signaling pathway begins with receptor tyrosine kinase activation and activation of phosphoinositide-3 kinase (PI3K) to make phosphatidylinositol 3,4,5-trisphosphate. This is followed by activation of the serine threonine

kinase PDK1, which phosphorylates AKT on its activation loop residue. A full activation of AKT requires a second kinase, the mammalian target of rapamycin (mTOR), part of the mTOR complex 2 (mTORC2), to phosphorylate the hydrophobic motif of AKT.

When fully activated, AKT has dozens to hundreds of targets, including the tuberous sclerosis (TSC) proteins. Some of these targets are important for mobilizing nutrient uptake receptors, and to bring in the second necessary component required for TSC inactivation, and to then fully activate mTOR.

The fact that AKT and all of its substrates need to be phosphorylated for full activation to happen has focused attention on the phosphorylation status of AKT at two sites in particular. Compared with its unphosphorylated version, AKT that has been phosphorylated on the activation loop by PDK1 shows a 100- to 1000-fold increase in activity. The second site phosphorylation by mTORC2 gives it an additional five-fold increase in activity.

Once AKT is fully activated and TSC2 is shut off, the energy-consuming point of the cell's life cycle — the initiation of protein translation and protein production — is initiated.

Promising inhibitors

mTORC1, the second complex that mTOR kinase is involved in, phosphorylates and activates S6K on its hydrophobic motif. That in turn phosphorylates the ribosomal protein S6, controlling ribosome biogenesis.

The second important substrate of mTORC1 is 4EBP1, the inhibitor of CAP-dependent translation. mTOR phosphorylates 4EBP1, removes it from the CAP and lets EIF-4G bind, leading to the translation of the most CAP-sensitive translation products, including proteins that are very important for cancer, such as cyclin D and MYC1.

This entire pathway has been studied extensively, and there are many compounds — including receptor tyrosine kinase inhibitors, PI3K inhibitors and AKT inhibitors — in phase 1 and phase 2 trials.

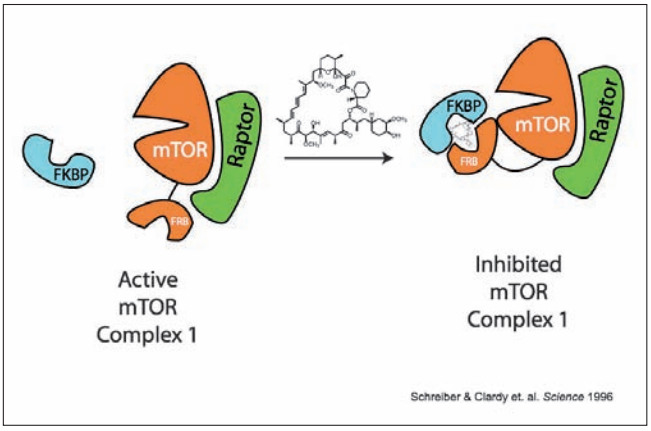

Figure 2
Rapamycin inhibits mTORC1 via the FRB domain.

One prominent inhibitor of one of these kinases is rapamycin, a natural product (see Verdine, page 105). Rapamycin and all the rapalogs have amazing promise, and are being heavily investigated as inhibitors at that key node of mTORC1.

Rapamycin inhibits mTORC1 in a way that only nature could have designed. It binds to a domain outside of the kinase domain called the FRB domain. When rapamycin binds to FRB and FKBP, the FRB Kinase Binding Protein, it makes a tri-molecular complex between FKBP, sandwiching around rapamycin, and then FRB. Through a mechanism that is poorly understood, this interaction inhibits mTOR's catalytic activity, or its ability to phosphorylate substrates.

This mode of inhibition is even more remarkable because it's highly selective. The second mTORC complex, mTORC2, phosphorylates the hydrophobic motif on AKT. Rictor, the defining compound of mTORC2, hides FRB from FKBP-rapamycin. So, rapamycin is a very selective inhibitor of mTORC1, and doesn't access this second complex[2].

In other words, rapamycin is a partial inhibitor of mTOR because it inhibits mTORC1, but not mTORC2.

Rapamycin and rapalogs should work in the same way because they rely on binding to FRB, which is only accessible in mTORC1 complex.

There are two problems with rapamycin, highlighting the few real deficiencies of a natural product. Rapamycin only partially inhibits the proliferation of most cells. It rarely kills cells, and inhibits cell growth and proliferation by about 40%, which is a disadvantage for a cancer drug.

Second, rapamycin induces a profound feedback effect on the pathway. At sub-nanomolar doses it induces phosphorylation on the PDK1 site as well as the mTORC2 site. With increasing doses of rapamycin, it becomes impossible to shut off the feedback because rapamycin cannot block the second complex, which phosphorylates the S473 site.

In a clinical trial for glioblastoma multiforme, some of the patients given rapamycin showed activation of AKT. Some patients responded to treatment, but in the non-responders, faster time to progression correlated with the induction of the feedback loop[3]. The idea of this feedback induction is becoming more recognized and studied.

Feedback loop

Although rapamycin is a powerful agent, it misses mTORC2 as a target. Some companies have discovered that compounds that inhibit both mTORC1 and mTORC2 also inhibit PI3K, indicating that they are multiplex inhibitors of the pathway. That's one way to short-circuit feedback.

Another approach is to develop a minimal targeted agent that would inhibit just the single kinase of the mTOR kinase domain, and assess whether it is a better inhibitor than is rapamycin. The goal is to go after the ATP-binding site, which must be accessible in both complexes because both phosphorylate substrates that require ATP binding.

From 300 kinase inhibitors developed over the years, after multiple cycles of a medicinal chemistry screen against mTOR and PI3K, there are two micromolar hits.

One of these is a compound called PP242, which has a pyrazolo pyramidine scaffold. It's an 8 nanomolar inhibitor of mTOR, but doesn't inhibit PI3K-α. Remarkably, it also inhibits 5 of the 220 invitrogen kinases, including PKC-α and PKC-β. The compound inhibits PKC-α at 50 nanomolar, which is troubling in terms of specificity, but it is nonetheless a very good inhibitor of mTOR. PP242 inhibits both mTORC1 and mTORC2, making it a better bet than rapamycin.

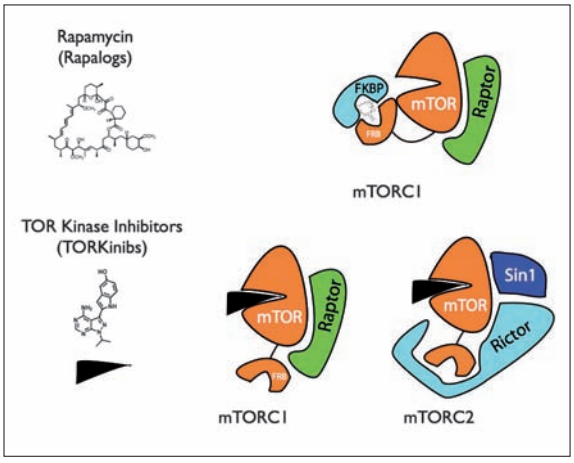

Figure 3
Distinct ways to target mTOR.

It's important when using a small molecule to study a kinase or a target that the experiments are designed to inform about the kinase, and not the small molecule itself. One way to ensure this is to take a second compound with overlapping specificity, but different structure.

In this case, the second molecule from the collection is a less potent inhibitor of mTOR, but it does not have any of the off-target effects observed with PP242. These two compounds together cover inhibition of mTORC1 and mTORC2, but they don't share PKC-α as a target.

This compound may also have off-target effects of its own, but because its structure is different, those effects are based on different motifs of recognition. When PP242 is added to 3T3-L1 cells, it potently inhibits phosphorylation of the S473 at the hydrophobic motif. This is a nice result because there has been controversy over whether mTORC2 is the kinase for that site, although genetic studies support the idea.

PP30, which is a PI3 kinase inhibitor, also blocks phosphorylation at S473. Surprisingly, at higher doses, there is also an effect of phosphorylation at T308, the PDK1 site, indicating a dose shift between inhibition at S473 and T308.

In vitro screening suggests that the compound does not inhibit anything upstream of the T308 site, including PI3K or PDK1. But to test this independently inside cells, a mutated AKT, in which the S473 phosphorylation site is replaced with an alanine, is transfected into cells.

When this mutated AKT is introduced, PP242 does not affect T308 phosphorylation, whereas the PI3K inhibitor does so completely, indicating that PP242 does not inhibit upstream of T308. The effect seen is because the loss of phosphorylation at S473 affects the T308 site indirectly.

Complete killing

PP242 differs from rapamycin in various ways. First, unlike rapamycin, which only partially inhibits proliferation of wild type mouse embryonic fibroblasts, PP242 and PP30 can kill cells completely.

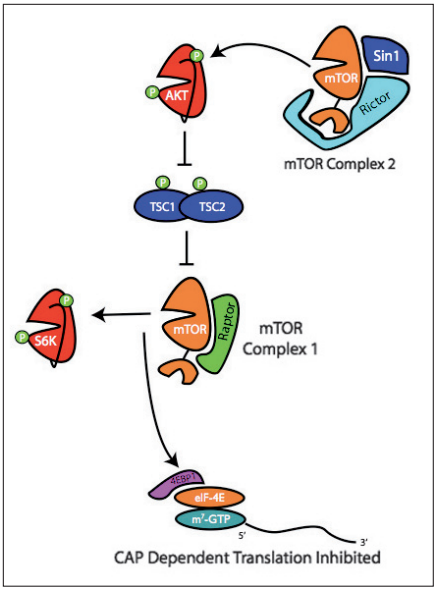

Figure 4
TORKinib is a more complete inhibitor of mTORC1 than is rapamycin.

PP242 and PP30 may be more effective because they are able to access and inhibit the second complex, which rapamycin cannot. But even in cells that have deletions in an essential component of mTORC2, the TORKinibs are still more potent inhibitors of proliferation than rapamycin.

These results suggest that going after the ATP site is more efficacious for an unexpected reason. The reliance on mTORC2 complex inhibition does not give better efficacy, so the focus shifts to mTORC1, even though rapamycin is a fantastic inhibitor of mTORC1.

In terms of the two downstream outputs, rapamycin and PP242 both inhibit the phosphorylation of 70S6K equally well. However, rapamycin does very little to inhibit 4EBP1 phosphorylation, whereas PP242 inhibits it over the same dose range as it inhibits S6K, suggesting that rapamycin is a more substrate-selective inhibitor of mTORC1 than initially appreciated. In contrast, the ATP-competitive TORKinib inhibits all outputs of mTORC1.

This is a surprising result, and a number of controls might be able to clarify whether rapamycin or the TORKinib fully blocks CAP-dependent translation better[4].

In the first control, a CAP pulldown assay, 4EBP1 will fall off the CAP-beads if it is phosphorylated, but should stay on if it is phosphorylated. As would be predicted, PP242 keeps 4EBP1 on the CAP beads. Rapamycin also does so to a small extent, but not at the level as the TORKinib.

Based on various CAP-dependent readouts with two different luciferases, there is a clear effect of PP242, but not of rapamycin, on CAP-dependent translation. There is no real difference between the two in terms of IRES-dependent translation.

Differential effects

Going back to the feedback loop, rapamycin has been shown to induce feedback of AKT. In HCT15 cells, low doses of PP242 also increase feedback to some extent, but increasing the dose of PP242 quenches the feedback. For these ATP-competitive inhibitors of mTOR, the potency and duration are very important.

One aspect of this growth factor signaling pathway is that it operates differently in different tissues. In fat cells, the effect is similar to that seen in tissue culture. Rapamycin does not inhibit 4EBP1 phosphorylation very well, but the TORKinib does so completely.

In contrast, in skeletal muscle, PP242 inhibits 4EBP1 as well as S6, but it doesn't inhibit S473 phosphorylation. This suggests that there may be a second kinase, or that mTORC2 is somehow differentially regulated in the skeletal muscle and is bypassing the ATP-competitive inhibitor. In the liver, rapamycin and PP242 are almost equally able to block 4EBP1 phosphorylation.

Finally, in a big screen of the TORKinib against more than 300 cancer cell lines, about 1-2% of the cells respond potently and another 5% less potently. There is no clear genotypic classification for sensitization, although PI3K mutations and PTEN loss are good, if not exclusive, determinants. KRAS is a poor determinant of sensitivity.

Mutations in PI3K and KRAS co-occur most frequently in colorectal cancer. Cell lines that are mutant for KRAS alone, but wild type for PI3K, are relatively resistant. But cells that harbor mutations in both KRAS and PI3K show significant sensitivity.

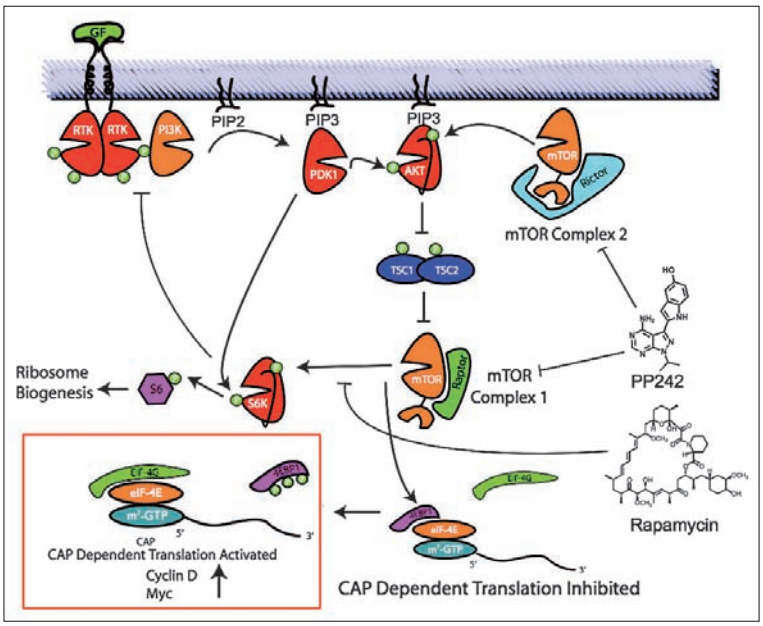

Figure 5
Summary of dual mTORC1+mTORC2 inhibition.

In both cell types, TORKinib blocks S473 phosphorylation, suggesting that the mTORC2 is the same in these two cells. With mTORC1, S6 is sensitive to the TORKinib, but interestingly, TORKinib blocks 4EBP1 only in the sensitive cells, and not in the resistant cells. This again points to the fact that 4EBP1 phosphorylation status is very important.

In summary, the new drug candidate adds efficacy because, unlike rapamycin, it also inhibits mTORC2 and blocks the rapamycin-induced feedback. But those observations do not explain the added benefits of fully blocking proliferation.

Because rapamycin only blocks one arm of the mTORC1 pathway, the other arm of the pathway may be more important. The fact that resistant cell lines seem to avoid the blocking of 4EBP1 phosphorylation also suggests that this pathway is important.

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Targeting the Hedgehog pathway in cancer

A report on a lecture by
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The Hedgehog (Hh) pathway is an ancient signaling cascade that directs patterning in most animals and is crucial for proper development. Although Hh signaling is very active during most stages in embryogenesis, it remains relatively quiet in adult life. However, aberrant reactivation of the pathway in adult tissue can lead to the development of cancer. Hh pathway activation in tumors such as basal cell carcinoma (BCC) and medulloblastoma is the result of inactivating mutations in PATCHED (PTCH) or activating SMOOTHENED (SMO) mutations. What's more, Hh pathway activation in other solid tumors, such as pancreatic or colon cancer is the result of up regulation of ligand expression in the tumor cells, which may act in a paracrine manner to activate the pathway in stromal fibroblasts. ***Frédéric de Sauvage*** *explained that targeting the Hh pathway with small molecule antagonists of the pathway therefore provides a new therapeutic opportunity for the treatment of both tumor types. GDC-0449, a systemic Hh pathway inhibitor, has been tested in a first-in-human, first-in-class, phase I study with locally advanced, multifocal or metastatic BCC patients. Anti-tumor activity is present in almost all BCC patients enrolled, thereby confirming the importance of inhibiting aberrant Hh signaling in BCC.*

Kinases are among the most popular targets in the drug discovery arena. However, there are no good kinases to target in some emerging pathways, such as the Notch, Wnt and Hedgehog (Hh) pathways. All the kinases in these pathways are promiscuous. These pathways are involved in the development of all tissues in which cancers — including breast, gastrointestinal tissue and skin cancer — develop.

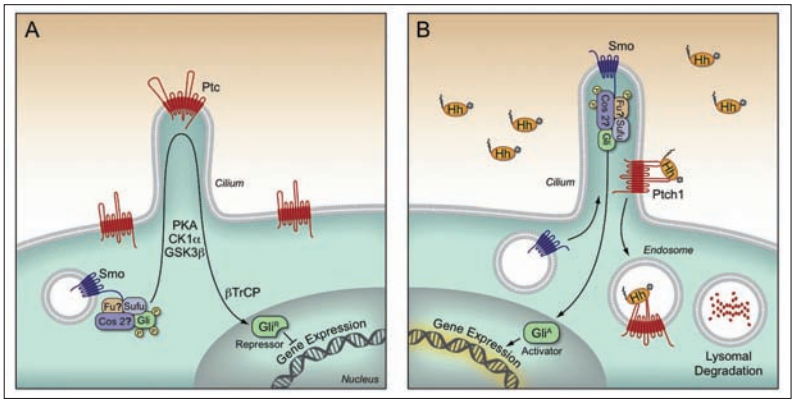

Figure 1
Aberrant activation of the Hedgehog signaling pathway can lead to cancer.

In the case of the Hh pathway, its signaling is crucial to the development of many organs. Mutations in this pathway lead to severe developmental disorders. This is the case for a developmental mutation in the Sonic Hedgehog (SHH) gene itself, which leads to the development of holoprocencephaly, a condition characterized by a dramatic loss of midline structure, and the presence of cyclopia in the offspring.

In contrast, a mutation in a negative regulator of the pathway, such as the GLI3 gene, leads to the development of such features as polydactyly. These two signs, cyclopia and polydactyly, are very common, and are useful ways to score phenotypes in mice when components of the pathway are knocked out.

The connection between Hh signaling and cancer was made about a dozen years ago, when a mutation

in the gene encoding Patched (PTCH) was identified in a patient with Gorlin's syndrome or basal cell nevus syndrome. This is a condition in which patients develop hundreds of basal cell carcinomas (BCCs) in their sun-exposed skin, and are at higher risk of development of other types of cancer, in particular medulloblastoma and rhabdomyosarcomas[1].

The Hh pathway is complex and still poorly understood. The Hh receptor PTCH is a negative regulator of Hh signaling and, in the absence of Hh, is present at the cell surface, in a structure called the primary cilium.

At the cell surface, PTCH represses the migration of Smoothened (SMO), which is present in intracellular vesicles, to the cell surface, thereby preventing SMO from signaling. When Hh comes along, it binds to PTCH and leads to the internalization and degradation of PTCH. That allows SMO to move to the cell surface, and in particular into the primary cilium, leading to the activation of the GLI transcription factor and its migration to the nucleus, where it activates transcription of Hh target genes.

In people with Gorlin's syndrome, in most sporadic cases of BCC, and in some sporadic medulloblastomas, there is a loss-of-function mutation in the PTCH gene. In the absence of PTCH, SMO migrates constitutively into the primary cilium and leads to constitutive activation of the Hh pathway.

The development of small-molecule antagonists targeting Hh pathway components downstream of PTCH would potentially be useful for the treatment of BCC or other cancers in which the pathway is mutated at the level of PTCH.

Pathway inhibitors

A natural compound called 'cyclopamine' was isolated from corn lilies, and was later shown to specifically target and inhibit the pathway at the level of SMO.

A screen for new inhibitors of the Hh pathway using the GLI-luciferase assay in a stromal fibroblast cell line has identified scaffolds that are more potent than cyclopamine. One specific scaffold called Hh-Antag was used to generate a small molecule that has since been moved into clinical development.

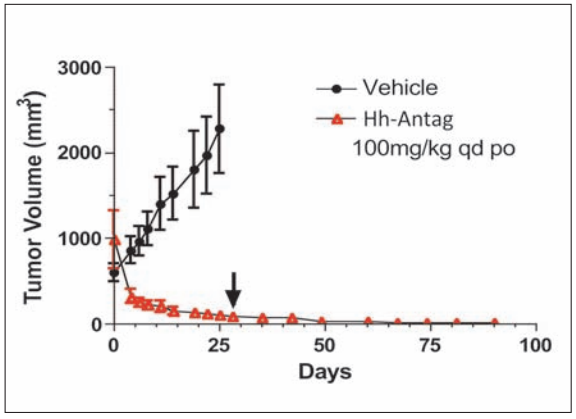

Figure 2
A large tumor treated for about three weeks with Hh-Antag completely regresses.

PTCH1 heterozygous mice, when crossed with p53 null mice, develop medulloblastoma with a 100% frequency. Treating these mice with Hh-Antag can completely cure medulloblastoma.

Tumor cells with mutations in the Hh pathway lose their dependence on Hh signaling for growth in tissue culture. In order to characterize Hh pathway inhibitors, an allograft model of $PTCH^{+/-}$-$p53^{-/-}$ meduloblastoma is used instead. In this model, a large tumor treated for about three weeks with Hh-Antag completely regresses and never regrows.

GDC-0449, the clinical lead, was tested in a phase I trial. The trial enrolled nine patients with locally advanced or metastatic BCC who were refractory to standard therapy. Analysis of archival tissue for GLI1 mRNA levels, a well-validated Hh target gene, confirm Hh pathway activity in these tumors.

In all nine patients, there is down regulation of GLI1 mRNA levels in skin biopsies; in seven of the nine, there is at least a two-fold down-modulation, and much higher than that in a few. There is no correlation between the level of down regulation and the level of the patient's response. Of nine patients, two have confirmed

partial responses, four have physical or clinical partial responses, two have stable disease and one patient has progressed.

The duration of the study ranged from 39 days to 438+ days. Most importantly, the toxicities, even during long treatment periods, are relatively mild and occur only after prolonged exposure. For these nine patients, the side effects were limited and included loss of taste, some thinning of hair, weight loss and salt imbalance.

Application of Hh pathway inhibitors is very promising, particularly in tumor types such as BCC and medulloblastoma, in which the pathway is activated by a mutation in upstream components. In the past couple of years, there has also been huge interest in another type of tumor, in which activation of the pathway is driven by ligand over expression.

Ligand activation

A large number of papers published in recent years have shown that many tumor types over express Hh ligand, which acts in an autocrine manner to activate the pathway in tumor cells.

However, there is no correlation between the cell line response and the level of expression of Hh target genes or pathway components, suggesting that the effect is not specific to Hh pathway inhibition. Of greater concern, the minimal amount of drug required for the inhibition of growth is extremely high, in the micromolar range.

The dose required is much higher than that needed to inhibit the pathway in a number of stromal lines that are able to transmit the Hh signal. At the concentration at which the pathway is fully inhibited, there is no effect on the growth of these lines. More important, at 10 micromolar cyclopamine or Hh-Antag, there is no regulation of the Hh target genes PTCH1 and GLI1. In comparison, cyclopamine and Hh-Antag effectively inhibit GLI1 in HPEM, a human fibroblast line.

In tumor types such as pancreatic adenocarcinoma (PDAC) in which the Hh ligand is over expressed, there is activation of the Hh pathway in the surrounding stroma, but not in tumor cells.

In a xenograft experiment, the Hh pathway is up regulated in the stroma in HPAFII cell lines, which express the Hh ligand, but not in the PL45 cell line, which does not express the ligand. Similarly, in a genetic model of PDAC, PdxCre driving K-RasG12D, Hh ligand expression is found in tumor cells, while the Hh pathway is activated in the surrounding stroma as measured by a PTCH-LACZ reporter gene.

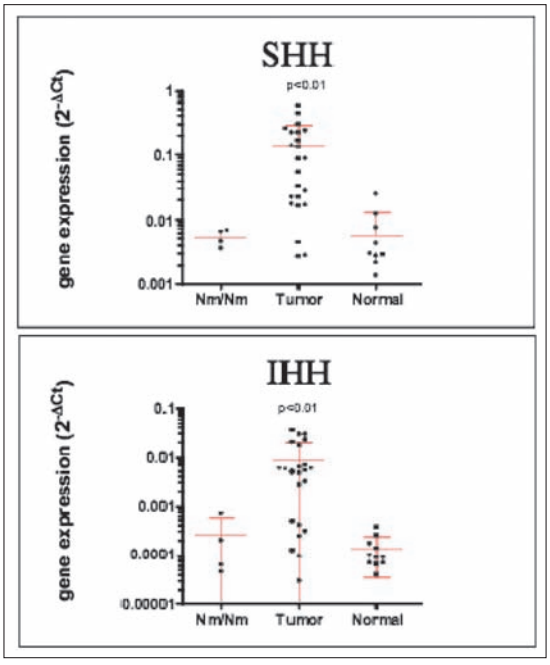

Figure 3
Hedgehog ligands are elevated in pancreatic adenocarcinomas.

To demonstrate that the epithelial compartment of the pancreas, either normal or from a tumor, is not competent to transmit the Hh signal, the SMO-M2 allele is crossed with PTCH-LACZ, together with PDX-CRE and KRAS.

In the resulting cells, there is clear expression of SMO-M2, but no activation or up regulation of β-gal at all in the tumor compartment. Again, there is only activity of β-gal in the surrounding stroma, indicating that these tumor cells cannot transmit the Hh signal[2].

This is consistent with a recent paper, in which researchers deleted SMO in this model using PdxCre, and showed that there is no effect or, if anything, a detrimental effect on the survival and tumor development in these mice[3].

Growth delay

These data all demonstrate that in tumors such as PDAC and colorectal cancer, the Hh pathway is activated in the tumor stroma, and not in the tumors themselves, so interrupting the communication between tumors and stroma could be of benefit.

Hh-Antag treatment delays growth in several primary tumors, generated by implanting pancreatic tumors directly from patients to recipient mice. After two passages in mice, the entire human stromal compartment in these models is completely replaced by mouse stroma.

Primers for Q-PCR that specifically recognize the human Hh target genes versus the mouse target genes show that Hh-Antag profoundly down regulates target genes in the mouse stromal compartment, but not the human compartment, confirming that the inhibition is in the stromal compartment.

By microarray analysis, beyond known Hh target genes, there are also gene signatures reflecting the secreted component of the Wnt pathway and the insulin growth factor pathway. These pathways have been related to the Hh pathway in a few other models, and could be responsible for mediating the Hh effect in the stromal compartment.

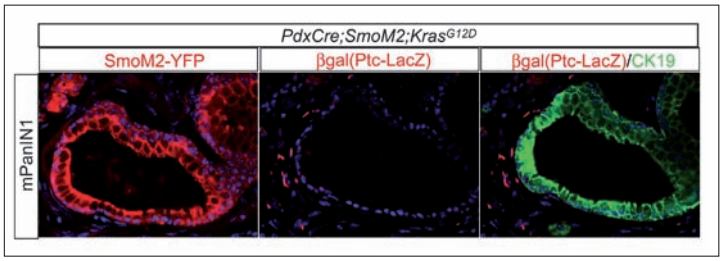

Figure 4
SmoM2 expression in pancreatic tumors does not activate the pathway.

Finally, the same data have been generated in colon tumors, with up regulation of Hh ligand expression in the colon tumors, but not in the normal adjacent tissue or in normal colon tissue. The same type of growth delay response and down regulation of Hh target genes in the mouse stroma is seen in primary tumor models. There is no effect on effect on body weight of the animal, suggesting that this is a well-tolerated treatment.

When the cell lines are profiled for the expression of Hh ligand, there is a wide range of expression, with some cell lines expressing large amounts of the Hh ligand, and some lines such as DLD-1 not expressing any. Xenografts established from these lines again show that Hh-Antag or pathway inhibitors can delay the growth of these tumors. The DLD-1 cell line, which doesn't express the Hh ligand, does not respond to pathway inhibition.

The data with Hh-Antag have been confirmed using 5E1, a well-known pan neutralizing antibody against Hh. The antibody generates the same amount of growth delay in two lines, HT29 and HT55, as is observed with the small molecule inhibitor[4].

Conditional knockouts

A conditional knockout of SMO in the stroma is generated using mice with a floxed SMO allele crossed with an inducible pCAGG-CreER allele. At day -15, the mice are shaved and treated with tamoxifen to induce the deletion. Two weeks later, hairs regrow on the control mice, but not on the SMO-deleted mice because Hh signaling is important for hair growth.

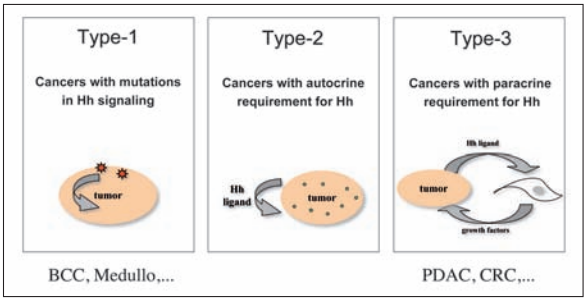

Figure 5
Models for Hedgehog activity in cancer.

At that time, the mice are implanted with HT29 tumor cells. About 2 or 3 weeks later, there is a growth delay in the tumors that are implanted in the SMO-deleted mice, compared with the control mice. The delay is very similar to the one that's observed with Hh-Antag treatment.

Finally, the experiment is done one additional way, which is to isolate mouse embryonic fibroblasts (MEFs) from two strains, either floxed SMO alone or CAGGCreER x SMOfl and treated *ex vivo* with tamoxifen to delete SMO. The cells are then treated *in vitro* with recombinant SHH to demonstrate that the SMO-deleted cells don't respond to Hh ligand stimulation compared with the control MEFs.

When these MEFs are mixed with HT29 cells and implanted into mice, SMO-deleted fibroblasts do not support tumor growth like control MEFs do. To make sure that the difference is not the result of a difference in growth in the fibroblasts, the HT29 cells are tagged with luciferase, and show that the difference is in growth of the tumor cells.

In summary, most cancers with mutations in the Hh pathway, or type 1 tumors, result from mutations at the level of PTCH1. Early clinical trials have shown that these tumors are likely to be sensitive to inhibition of the Hh pathway.

Another type of tumor, the type 3 tumor, also exists, with a paracrine requirement for Hh pathway activity. The tumor cells make Hh ligand, which stimulates the pathway in the tumor stroma. In return, the stroma provide a beneficial environment for the tumor's growth. The nature of that environment is yet to be characterized.

No individual factor has been proven to be responsible for mediating the Hh effect. But it is possible that in addition to factors affecting tumor cells directly, stimulation of the stroma provides factors such as the pro-angiogenic factor vascular endothelial growth factor, which has been shown to be downstream of Hh stimulation in fibroblasts. There may be multiple ways in which paracrine activation of the Hh pathway may be beneficial to the tumor.

Targeting the tumor stroma has important consequences for the use of Hh pathway inhibitors in the clinic. Inhibitors alone may not be able to regress type 3 tumors, but have to be considered in combination with agents that directly target the tumor cells.

Finally, the existence of type 2 tumors cannot be ruled out. These are tumors in which the Hh ligand stimulates the pathway in the tumor cells themselves in an autocrine manner. However, none of the data generated using very high concentrations of Hh pathway inhibitors provide any evidence for the existence of these tumors.

It's possible that the Hh pathway is important for cancer stem cells. But when entire tumors are profiled, it would not detect very rare tumors or stem cells that would respond to Hh pathway inhibition.

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Targeting histone deacetylases in cancer

A report on a lecture by
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Epizyme, Inc., Cambridge, USA

Histone deacetylase (HDAC) inhibitors represent a promising new class of anti-tumor agents. HDACs are enzymes that catalyze the removal of acetyl groups from the lysine residues of proteins, most notably from the core nucleosomal histones. Four classes of HDACs have been identified. Histone acetyltransferases have been found to be mutated in a variety of cancers, while HDACs have been shown to be over expressed, aberrantly recruited to oncogenic transcription factors and mutated in cancer. HDACs thus represent potential targets for small-molecule inhibitors, and HDAC inhibitors are being evaluated as cancer therapies. Vorinostat is the first HDAC inhibitor approved for the treatment of cancer. Vorinostat has been found to inhibit proliferation and induce apoptosis or autophagy of cultured transformed cell lines derived from several different cancers, although the molecular mechanisms have yet to be fully clarified. ***Victoria Richon*** *presented data suggesting that vorinostat-induced accumulation of acetylated histones is not sufficient to predict response to vorinostat and that the survival pathways in those cells modify the vorinostat response. Further investigation of vorinostat in other hematologic malignancies and solid tumors is ongoing.*

Histone deacetylases (HDACs) are enzymes that catalyze the removal of the acetyl group from the lysine residues of proteins. In some ways, HDAC is a misnomer because more and more proteins have been found to be reversibly acetylated. The opposing enzymes are histone acetylasetransferases (HATs).

There are four classes of mammalian HDACs that can be classified based on the structure of their catalytic domain. Class I, II and IV HDACs are similar, whereas the class III enzymes have evolved a different catalytic domain. None of the HDAC inhibitors in development for cancer target the class III enzymes.

There are 11 class I, II and IV HDACs that are again divided based on their catalytic domain. Class II HDACs are further subdivided based on their structure. The IIA HDACs have a long amino terminus and the catalytic domain, whereas the IIBs have two catalytic domains.

HDACs have been shown to be over expressed in certain human cancers. They are recruited by oncogenic transcription factors. In contrast, HATs are translocated and mutated in cancer, leading to loss of acetylation in tumors.

The strongest evidence that has propelled this field is the development of HDAC inhibitors, even before the first HDACs had been cloned in 1996. These inhibitors can be divided into different structural classes: short-chain fatty acids, hydroxamic acids including vorinostat, cyclic tetrapeptides and benzamides.

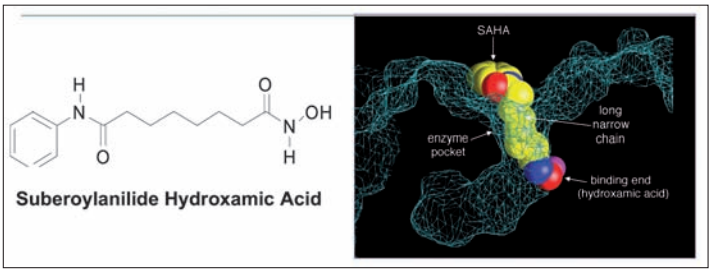

Figure 1
Vorinostat was the first HDAC inhibitor to be approved for the treatment of cancer.

Vorinostat or SAHA was the first HDAC inhibitor to be approved for the treatment of cutaneous T-cell lymphoma

(CTCL). All HDAC inhibitors, including Romidepsin or depsipeptide, which was filed for approval in late 2008, have shown activity in CTCL.

Vorinostat was identified in a cell-based screen for agents that induce differentiation. It was later found to be an inhibitor of HDAC activity, with an IC50 of about 50 nM. In 2006, the Food and Drug Administration approved vorinostat for the treatment of advanced CTCL. In combination with other agents, the drug has also shown activity against a variety of solid tumors.

Although vorinostat is usually called a pan-HDAC inhibitor, it doesn't inhibit all family members. Based on enzyme assays for the different HDACs, vorinostat inhibits HDAC1, 2, 3 and 6, but not 4, 5, 7, 11 and also perhaps HDAC9.

Antibodies for acetylated histones show that vorinostat is not selective: when cells are cultured with vorinostat, there is accumulation of acetylated histones against all of the core nucleosomal histones.

By ELISA assay, raising the concentration of vorinostat in a bladder carcinoma cell line results in accumulation of acetylated histone H3, which correlates with the drug's anti-proliferative activity in this system.

Across a broad panel of 200 cell lines from four tumor types — lymphoma, colon, breast and lung cancer — no one cell type is more sensitive to the anti-proliferative effects of vorinostat than others, with the exception perhaps of lymphomas.

At 1 micromolar concentration of vorinostat, a substantial number of lymphoma cell lines are very sensitive to vorinostat compared with any of the other tumor types. This is about the concentration that is achievable in patients with a continuous dosing schedule, suggesting that as a single agent on this schedule, vorinostat is not predicted to have much activity against these tumor types, except in a subset of lymphomas.

Vorinostat's anti-tumor activity can also be seen *in vivo* in a variety of xenograft models as well as in carcinogen-induced models.

Inducing apoptosis

The □-myc lymphoma transgenic model is a nice experimental system because there is a tumor bank of the different cells. Effects of the drug can be observed both *in vitro*, and *in vivo* after transplantation into mice.

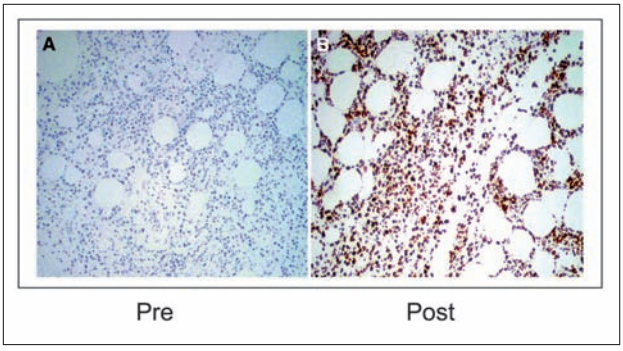

Figure 2
Vorinostat causes accumulation of ac-Histone H3.

In the *in vitro* studies, a 0.5 micromolar concentration of vorinostat induces accumulation of acetylated histones and apoptosis, either measured by annexin or by accumulation of sub-G1 cells.

Vorinostat treatment *in vivo* also increases apoptosis, as measured by the percentage of cells that are in sub-G1. Mice treated with vorinostat all survive during the treatment period compared to vehicle-treated animals. However, when treatment is stopped, the lymphoma relapses and the mice die, indicating that vorinostat does not cure the cancer.

The increase in apoptosis is specific for the lymphoma cells. If the lymphoma cells that have the CD45.2 antigen are transplanted into a mouse that has CD45.1, after eight hours of treatment with vorinostat, there is a very large decrease in those lymphoma cells, without affecting the normal compartment. This indicates that vorinostat selectively induces apoptosis in the lymphoma cells.

Vorinostat has a therapeutic effect in wild type E□-myc lymphoma line, but expressing BCL-2 in the cells essentially blocks the ability of these cells to undergo apoptosis. Crossing with a mouse lacking p53 has no effect, however.

More information about the response in the lymphoma model suggests that vorinostat's ability to cause the accumulation of acetylated histones is required, but not sufficient, for response. The survival pathways in those cells regulate the ability to induce apoptosis. Over-expression of BCL-2 blocks the ability to undergo apoptosis following vorinostat treatment. However, loss of p53 does not impair the ability of vorinostat to induce apoptosis.

Vorinostat is being tested in more than 80 different clinical trials. A phase I trial at Memorial Sloan-Kettering Cancer Center in New York showed that vorinostat has linear pharmacokinetics, and good bioavailability in patients with advanced cancer[1].

The maxiumum-tolerated once-daily dose is 400 mg given orally administered on a continuous basis. There is preliminary evidence of clinical activity in a broad range of solid tumors, with complete response in diffuse large B cell lymphoma, three partial responses including one in diffuse large B cell lymphoma, one in laryngeal and one in thyroid cancer, and two unconfirmed partial responses in mesothelioma.

Chronic administration is feasible; 22 patients were in a study for between 4 and more than 37 months. One patient has been administered the daily dose for more than four years. The most common toxicities are fatigue, gastrointestinal distress, hyperglycemia, hypokalemia, anemia and thrombocytopenia. And the dose-limiting toxicities are similar between patients with solid and hematologic malignancies.

Stable disease

At the lowest dose of 200 mg per day in one study, following two hours of treatment, histone acetylation can be detected in peripheral blood mononuclear cells (PBMCs) from patients[1].

As the drug dose is increased, there is an increase in the duration of acetylation of histones in PBMCs. The data suggest that at the maximum tolerable dose of 400 mg, the patients see a pulse of acetylation with a duration of approximately eight hours per day. At a 600 mg once-daily dose (which is above the maximum tolerated dose), there are high levels of acetylation for over 12 hr per day.

The same trend can also be seen in biopsies of the tumor. At the 400 mg dose, four hours post-dose, there is an accumulation of acetylated histones in the biopsy from a lymph node of a CTCL patient[2]. In a glioblastoma trial in which patients are dosed before surgery, acetylated histones accumulate in the biopsy, showing that vorinostat is able to cross the blood-brain barrier.

Many patients have been on vorinostat for two to three years, suggesting that the drug can result in stable disease. Many mesothelioma patients have benefited from the drug, which led first to a phase II trial and now to a phase III mesothelioma trial.

In the CTCL registration study, about 30% of patients with treatment-refractory advanced CTCL showed at least a partial response to the drug. The median time to response was relatively short, at less than two months, and there were clinically meaningful long-lasting responses. Once again, the 400 mg dose of vorinostat was well tolerated.

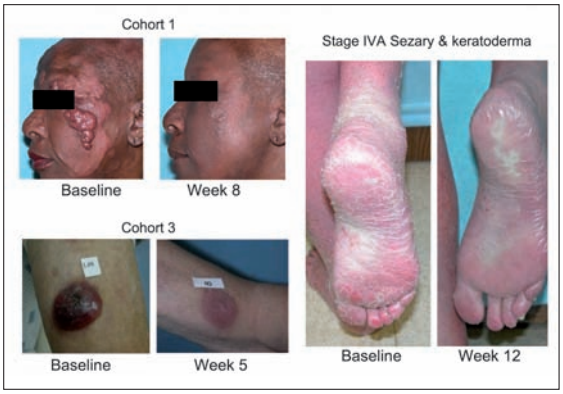

Figure 3
Treatment with vorinostat can result in stable disease.

The conclusion from the study, which formed the basis for approval, was that vorinostat is an effective single agent for CTCL, and may also find utility in a combination regimen with other cutaneous or systemic agents.

Pre-clinical studies with the lymphoma panel of cell lines may help identify why only 30% of the patients responded in the trial. Baseline profiling in all 200 of the cell lines helped identify sensitivity or resistance markers in the patients.

In the lymphoma cell line, there is no correlation between response to vorinostat and HDAC and HAT expression, multi-drug resistance pumps or anti-apoptotic proteins such as BCL-2, BCL-XL and survivin. In some cases BCL-2 (as in the E□-myc lymphoma model) can cause resistance.

STAT-associated genes are expressed at much higher levels in cells that are resistant to vorinostat. The cell lines that are most sensitive to vorinostat have much lower expression of STATs and, in some cases, lower activation of STATs as measured by phosphorylation.

Synergistic effects

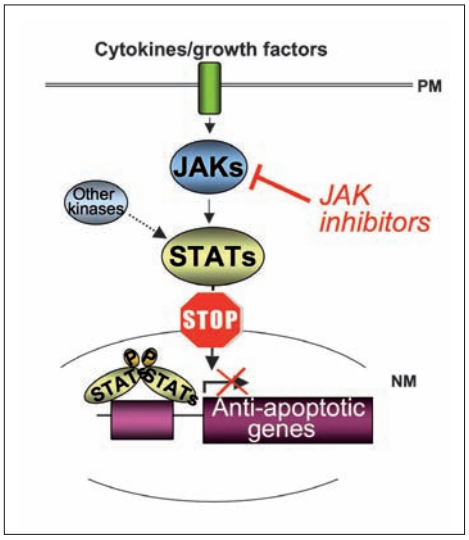

Figure 4
Combining JAK inhibitors with vorinostat has a synergistic effect.

Combining vorinostat with a JAK inhibitor, which blocks the STATs, results in a loss of cell viability. The JAK inhibitor and vorinostat are clearly synergistic.

Data from baseline skin biopsies of 43 patients shows that patients who have a nuclear localization for STAT1 have a significantly higher chance of not responding to vorinostat treatment. Similarly, patients with high levels of P-STAT3 have a significantly higher chance of not responding to vorinostat.

Here again, the effect is not dependent on the ability to acetylate the histones, or on inhibition of HDAC activity. In samples with high levels of STAT, the drug is instead unable to induce apotosis.

These observations are helpful in deciding which combinations of drugs to use for treating cancers. In CTCL, for example, combining a JAK inhibitor and vorinostat would be useful[3].

Vorinostat is known to inhibit HDAC6 activity at equal concentrations to HDAC1, 2 and 3. A recent study has shown that HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress[4].

This is interesting because cells treated with bortezomib can avoid apoptosis by forming aggresomes. Bortezomib alone induces some apoptosis, and treatment with vorinostat alone has no effect, but when bortezomib and vorinostat are combined, apoptosis is enhanced, and these large aggresomes do not form[5].

There have already been two phase I trials of vorinostat in combination with bortezomib in patients with relapsed or refractory multiple myeloma, showing results similar to those seen in pre-clinical studies.

The results are encouraging because most patients in these studies benefited from the combination treatment, showing a partial response, minor response or stable disease; only two patients saw progressive disease. Understanding more about the specific HDAC enzymes — HDAC6 in this case — and their function can thus help launch trials based on mechanistic rationales.

To summarize, vorinostat inhibits HDAC1, 2, 3 and 6 activity. There are transcriptional and non-transcriptional

effects that can lead to apoptosis. Depending on the cell type and the combination, different effects can play a role in the response.

Vorinostat, through inhibition of HDAC6, is thought to cause acetylation of HSP-90, although that needs to be confirmed. Resistance to vorinostat doesn't seem to be at the level of its ability to inhibit enzymes, or even perhaps to induce these downstream effects, but seems to be dependent on whether there are survival pathways in the tumor cells that can block apoptosis.

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PART V: Strategies for drug discovery

Metabolomic profiling of prostate cancer progression

A report on a lecture by
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Howard Hughes Medical Institute, Ann Arbor, USA

Combining metabolomics with genomics and proteomics data can help better understand events during prostate cancer progression. Metabolites involved in epigenetic processes are enriched in prostate cancer progression. Sarcosine is a key metabolite induced in prostate cancer progression and is associated with cancer cell invasiveness and aggressiveness. ETS gene fusion-induced cell invasion is associated with an increase in sarcosine, suggesting a possible genetic basis for the elevation. Sarcosine appears to be regulated by both androgen and ETS gene fusions, and may represent a metabolomic biomarker of aggressive forms of prostate cancer. Sarcosine needs to be analyzed in the context of other metabolites picked up through an unbiased screen in order to develop a panel of metabolites for prostate cancer aggressiveness. ***Arul Chinnaiyan*** *described one of the first unbiased assessments of metabolites in cancer development.*

Unbiased metabolomics profiling of cancers can be combined with other '-omics' approaches to study cancer progression, and build a more comprehensive view of the molecular alterations that occur during cancer development. The model used in this case is prostate cancer, although other cancers can also be studied in a similar manner.

Prostate cancer is most common non-cutaneous malignancy among men in the Western world. Biomarkers for prostate cancer have some deficits: for example, screening for the prostate specific antigen, or PSA, has only 20% specificity.

One crucial issue in prostate cancer is trying to distinguish between the aggressive tumors that metastasize and kill the patient versus the indolent slow-growing tumors that most patients have. Patients are often over-treated because there are no good ways to distinguish between these two types of tumors.

There is a histo-pathologic progression of prostate cancer from benign epithelia to prostatic intraepithelial neoplasia, thought to be the precursor lesion. Clinically localized disease moves on to become metastatic, spreads to different parts of the body, and eventually becomes hormone-refractory disease.

The different molecular alterations during progression to prostate cancer can be grouped into different pathways and networks, including androgen signaling, glutathione metabolism and ETS transcription factors.

Various research groups have developed different platforms to measure each of these types of alterations in matched tumors, looking at genetic, genomic,

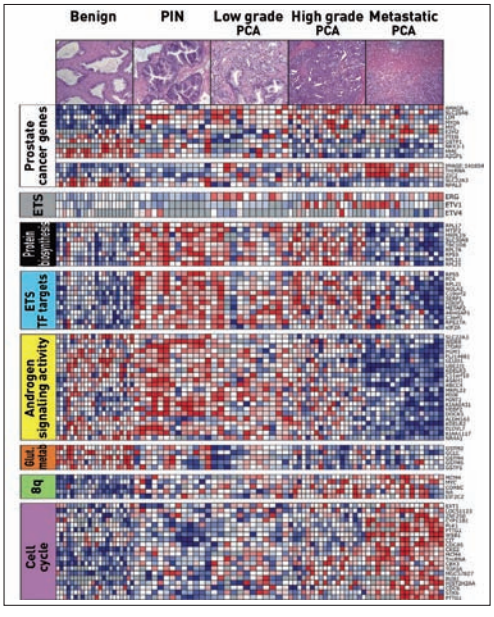

Figure 1
A molecular concept map of prostate cancer progression.

transcriptomic, and proteomic alterations. These alterations appear to expand out at the level of proteomics.

One area that's been relatively unappreciated is the study of small molecules or biochemicals — essentially, metabolomics. The human metabolome comprises about 3,000 compounds, so the expansion in molecular alterations at the level of proteins is condensed again at the level of metabolites, which are a more digestible set of alterations to study.

Metabolites are also conserved through species, and end up being the final common functional readout of both normal biology and human disease. Distinct sets of genes, proteins and metabolites all dictate prostate cancer progression.

The impetus of this study is to decipher the molecular networks that distinguish organ-confined prostate cancer from metastatic disease, perhaps leading to the identification of biomarkers of invasion and disease aggressiveness. Considerable work has been done on the role of gene and protein expression to understand prostate cancer biology, but much less has been done on metabolomic alterations that appear to be the distal read-out of disease pathophysiology.

Metabolism and cancer

Metabolomics is the 'omics' science of metabolism. It is the only window into the current and actual state of the cell at a specific point in time, and seeks to capture physiological status as a function of biochemical activity.

There are a number of reasons to study these alterations in the context of the other molecular alterations. Metabolites are highly conserved and highly defined, which make them computationally approachable, more amenable to interpretation, and more precise in their characterization. Yet, as part of a larger whole, metabolomics datasets can be integrated with the other 'omics' datasets[1].

The role of metabolism in cancer is not a new concept. First suggested by Otto Warburg many years ago, metabolism is the basis of the 'Warburg effect', which holds that most cancer cells use glycolysis followed by lactic acid fermentation in the cytosol even in the presence of oxygen.

Cancer cells also undergo oxidation of pyruvate in their mitochondria in the presence of oxygen. Warburg thought this was a cause of cancer, but others have suggested that this is instead a consequence of cancer. Some studies — for example, that the M2 spliced form of pyruvate kinase is turned on specifically in cancer cells — have gone on to explain aspects of the Warburg effect.

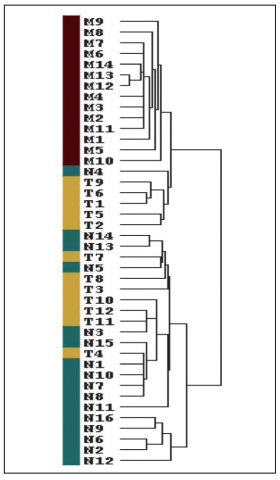

Figure 2
Metabolomic profiling of prostate cancer.

This study focuses on metabolite alterations in the context of biofluids including tissues, urine and plasma. The goal is to identify metabolomic signatures during prostate cancer development and progression; pinpoint metabolites that could be used in multiplex to detect and monitor prostate cancer in tissues and biofluids; and integrate genomics, proteomics and metabolomics data to better understand events during prostate cancer progression[2].

High-throughput liquid and gas chromatography-based mass spectrometry (GC-MS) is used to monitor more than 1,265 metabolites across 262 clinical samples — including tissue, urine, and plasma — related to prostate cancer.

Metabolites are extracted from frozen sections taken from benign clinically localized and metastatic prostate cancer. Using either GC-MS or LC-MS, the metabolites are analyzed, normalized and subjected to bioinformatics interpretation, which matches metabolites to different pathways and different gene expression alterations.

By Venn diagram analysis of the metabolomic coverage across different biospecimens, there are very few metabolites that are shared across the specimens. This is expected because the samples, which are derived from prostatic tissue, plasma and urine, are quite different.

Drilling down into the tissues themselves, there are many metabolites that are shared across tissues, from benign to clinically localized prostate cancer and metastatic disease.

Metabolomic signatures

The data can be represented either in a way that's analogous to heat maps, or to a Z-score plot, where the data is normalized against benign adjacent samples. In clinically localized samples, there are some log-fold changes in metabolites. On the Z-score plots, the metabolic networks can be seen to get extremely dysregulated with progression to metastatic prostate cancer.

As is done in gene expression studies, the samples can be classified into metabolomic signatures in an unbiased hierarchical clustering, which cleanly segregate out the metastatic prostate cancers. The benign and clinically localized tumors are more difficult to distinguish, but they also tend to segregate to some degree. The metastatic data also segregate out from the localized cancers and the benign samples by principal components analysis.

As would be expected, the metabolic alterations fall into classic pathways, including purine and pyramidine metabolism and amino acid metabolism. Pathways that are robustly dysregulated across prostate cancer progression include amino acid metabolism, as well as methylation and methyltransferase pathways.

Molecular concept network maps show the relationship of clinically localized prostate cancer to different amino acid metabolism pathways that are enriched, and some of the methylation pathways that appear to be perturbed through progression to metastatic disease.

One of the metabolites that fulfills both the characteristics of being in the amino acid metabolism pathway as well as being in the methylation pathway is a modified amino acid called sarcosine, which is essentially a methylated version of glycine. Sarcosine is the most increasingly elevated metabolite in the progression from benign to clinically localized to metastatic disease, but there are about 10 other metabolites that fulfill similar criteria.

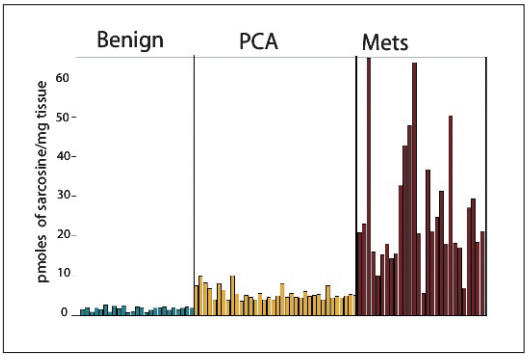

Figure 3
Validation of sarcosine elevation in prostate cancer.

Glycine can be converted to sarcosine by the enzyme glycine N-methyl transferase (GNMT). The enzyme sarcosine dehydrogenase (SARDH) can in turn convert sarcosine to glycine.

Using an independent set of prostate cancer tissues and a new highly sensitive GC-MS based assay shows that sarcosine is extremely elevated in metastatic prostate cancer, and incrementally elevated in clinically localized disease relative to benign adjacent tissues, essentially validating the unbiased metabolomic MS-based approach used initially.

In order for this metabolite to be used as a biomarker in prostate biopsies, it needs to be used non-invasively. Indeed, in post digital rectal exam urine, sarcosine is consistently elevated in the sediment of men with prostate cancer, as well as in the supernatant.

Using a prospectively collected cohort of men with an elevated PSA, if men are separated into those that

are then biopsy-positive and biopsy negative, biopsy-negative men may also have prostate cancer. But sarcosine levels are elevated in those who have cancer, even in those that are biopsy-negative.

To evaluate sarcosine as a biomarker, it has to be compared to a standard test, which, in this case, is the unreliable PSA test. In that context, sarcosine adds information, and is more promising than is PSA, albeit not perfect. But it may be possible to develop a panel of metabolites that would include sarcosine to supplement PSA screening.

Sarcosine's effects

Sarcosine may be associated with cancer cell aggressiveness because its levels are highly elevated in metastatic prostate cancer even relative to clinically localized disease.

When primary prostate epithelial cells, or RWPE benign immortalized prostate epithelial cells are examined, benign cells have low sarcosine production, whereas prostate cancer cell lines have elevated sarcosine levels. This also correlates with invasiveness of the cells based on Boyden-chamber type matrigel assays.

A majority of prostate cancers harbor a gene fusion or a translocation, whereby there's an androgen-regulated genetic element, usually the gene TMPRSS2, fused upstream of a family of oncogenic ETS transcription factors[3].

Is there a genetic basis for the sarcosine elevation? Some previous studies have shown that when the gene fusion product is introduced into benign prostatic epithelial cells, the cells become highly invasive in matrigel-type assays. When these gene fusions are introduced into benign RWPE cells, for example, they induce invasiveness, but also increase levels of sarcosine compared with controls.

As another control for this experiment, VCaP cells, taken from patients with metastatic prostate cancer, harbor the gene product TMPRSS2-Erg, also express high levels of sarcosine, and are invasive. When the gene fusion product is knocked down using RNAi, it blocks their invasiveness, as well as their ability to increase sarcosine levels.

The idea behind studying metabolites is to find biomarkers for aggressive disease that don't necessarily function in any of the pathways of prostate cancer progression. Interestingly, however, when sarcosine is added to benign cells, either RWPE cells or primary prostatic epithelial cells, these cells become invasive. Other control amino acids, including alanine, do not have this effect[4].

Sarcosine not only induces invasiveness, but also increases cell motility relative to other amino acids such as alanine. But it does not affect cell proliferation or cell cycle, as assessed by flow cytometry and cell count assays, at least in the short term.

Glycine has an intermediate effect relative to a number of other amino acid controls. The potential explanation for that is that the enzyme GNMT can convert glycine to sarcosine.

SARDH can degrade sarcosine back to glycine. There are also some side pathways, such as an enzyme that can convert dimethyl glycine to sarcosine. Perturbing some of these enzymes that lead to sarcosine can be one way to study these pathways further.

Androgen signaling

When GNMT is knocked down, it decreases the invasive capability of the cancer cells, but also markedly decreases the sarcosine generation in these cells. The cells maintain their invasive ability when glycine is added to them.

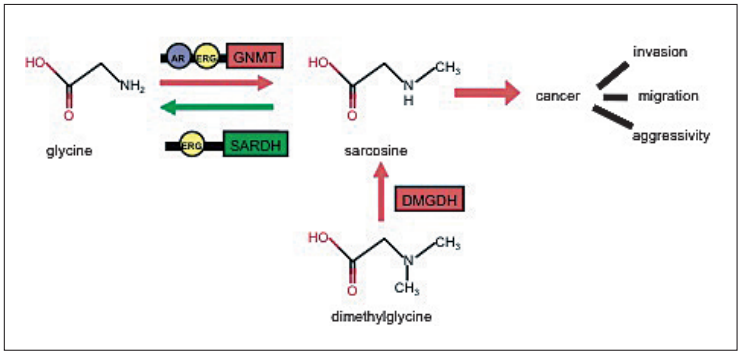

Figure 4
The role of the sarcosine pathway in prostate cancer progression.

The converse experiment is to take benign prostate epithelial cells and knock down SARDH, which degrades sarcosine back to glycine. When SARDH is knocked down, sarcosine levels accumulate in these cells and induce cell invasiveness.

Androgen receptor and androgen signaling are known to be important mediators of prostate cancer development, and a target for prostate cancer therapy. RT-PCR studies show that androgen treatment of cancer cells induces GNMT, and represses SARDH.

CHIP assessments of the androgen receptor as well as the ERG gene fusion product reveal that both are recruited to the promoter enhancer element of GNMT, whereas the ERG gene fusion product is recruited to SARDH.

To determine how sarcosine mediates the cancer cells' invasive potential, the cells are profiled by gene expression analysis. The analysis suggests that adding sarcosine to benign prostate epithelial cells phosphorylates EGFR, and activates downstream readouts of EGFR signaling.

Small molecule inhibitors against EGFR signaling, including erlonitib, block EGFR activation by sarcosine. Adding erlonitib blocks sarcosine-induced invasion, suggesting that sarcosine potentially mediates its effects through EGFR signaling.

Other inhibitors against EGFR signaling, including a small molecule inhibitor, a blocking antibody against EGFR, as well as two independent siRNAs against EGFR all block sarcosine-induced invasion. These observations suggest a link to EGFR signaling downstream of sarcosine-mediated invasion.

The results also raise several questions, including the clinical implications of this finding. The work may lead to a metabolomic biomarker panel for the early detection of prostate cancer in urine and, more importantly, a metabolomic panel to predict the aggressivesness of prostate cancers.

Mapping some of these metabolic alterations may also help perturb these metabolite profiles to have a therapeutic effect. The enzymes that lead to sarcosine, as well as sarcosine itself, could be potential targets for prostate cancer.

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Synthetic lethal interaction between oncogenic KRAS dependency and suppression of STK33 in human cancer cell lines

A report on a lecture by
Gary Gilliland
Harvard University, Boston, USA

RAS is the most commonly mutated gene across a broad spectrum of human cancers, but despite several decades of effort, no clinically viable therapeutic modalities that target oncogenic RAS alleles have emerged. An alternative to direct targeting of oncogenes is to perform 'synthetic lethality' screens for genes that are essential only in the context of specific cancer-causing mutations. Synthetic lethality refers to a lethal interaction between two mutant alleles, neither of which is sufficient to confer lethality on its own, but are lethal in combination. Using high-throughput RNA interference in cell lines harboring either mutant or wild type KRAS, suppression of STK33 has been shown to preferentially inhibit the viability and proliferation of cells that are dependent on mutant KRAS. Biochemical analyses support the hypothesis that STK33 promotes cell growth and survival by regulating the activity of S6K1 selectively in mutant KRAS-dependent cells. Based on these observations, ***Gary Gilliland*** *described STK33 as a target for the treatment of the broad spectrum of mutant KRAS-driven cancers.*

Synthetic lethality screens are one approach for identification of vulnerabilities induced by cancer-associated mutations that could serve as therapeutic targets. The term 'synthetic lethality' describes a situation in which alteration of two genes results in cell death, whereas alteration of either gene alone is compatible with cell viability.

Work in yeast and fruit flies has revealed that these are sometimes genes in the same essential pathway, or they can be genes in parallel pathways that cooperate with regard to an essential function, but may not have any other interface. They can also be genes in distant pathways that become functionally connected because of the response of the cell to a specific perturbation, such as an oncogenic mutation.

More recent studies have extended these observations to mammalian cells. Based on these insights it should, in principle, be possible to perform synthetic lethality screens in human tumor cells, using tools such as RNA interference, in order to identify liabilities induced by oncogenes for which targeted therapeutic agents are lacking.

Members of the RAS gene family are the most frequently mutated oncogenes in human cancers. Mutations in KRAS are represented in a broad spectrum of tumors, including up to 10% of myeloid leukemias.

Synthetic lethal screens may be able to identify druggable targets in the context of KRAS, which has traditionally been considered an undruggable target[1]. Inhibition of farnesyl transferase, which catalyzes the farnesylation of KRAS required for its activity, is one reasonable idea to target KRAS. However, among other challenges, this almost invariably results in escape through other prenylation mechanisms.

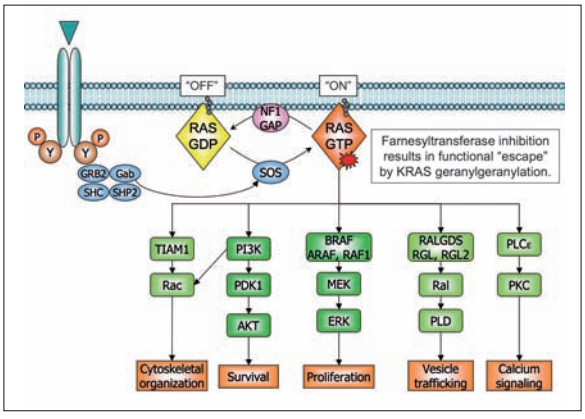


Figure 1
Oncogenic KRAS as an "undruggable" target.

To identify alternative therapeutic target genes that are essential for cancer cell viability in the context of mutant KRAS, high-throughput loss-of-function RNAi screens have been performed initially in two human acute myeloid leukemia cell lines, NOMO-1 and THP-1.

NOMO-1 is derived from a patient with acute monocytic leukemia, and has a KRAS G13D mutation as well as a 9:11 translocation involving the MLL-AF9 fusion oncogene, and mutations in p53 and p16. THP-1 is nearly identical except that it has an activating mutation in NRAS.

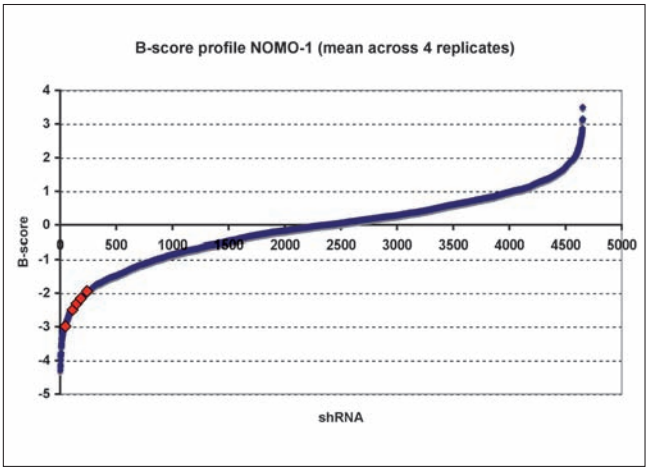
The negative selection arrayed screen utilizes a kinase-enriched subset of the Broad Institute's TRC shRNA Library, which has 5024 hairpins targeting 1011 genes at a five-fold redundancy for each of those genes. The library includes all kinase genes, phosphatase genes and select known cancer-related genes, including RAS family members[2].

Scoring hits

The experimental design is straightforward. The cell lines are seeded and transduced with lentivirus containing the respective shRNA and a puromycin selection marker. Stably transduced lines are selected with puromycin for 5 days and then viability measured at day 7.

The B-score profiles — Z scores with a computational score to compensate for edge effect on the 384 well plates — for the cell lines read out the effect of a given hairpin on cell viability. Applying a B-score threshold of median minus one standard deviation identifies 855 and 663 scoring shRNA for NOMO-1 and THP-1, respectively.

Figure 2
Top hit in NOMO-1: KRAS.



The next important metric is to define a gene as a "hit" only if at least two different shRNAs have B-scores below the threshold. With those criteria, NOMO-1 has 212 hits and THP-1 has 129, which is a still a challengingly high number for an academic lab to validate and biologically evaluate.

One encouraging result is that the top hit for NOMO-1 is KRAS itself, indicating that these cells are dependent on KRAS for their survival, and validates the screening platform. NOMO-1 cells could therefore be useful for identifying genes essential for cell viability in the context of oncogenic KRAS.

Other investigators using the same kinase-enriched library at Broad have screened 10 additional cancer cell lines in different tissue types, of which 5 are known to be mutant for KRAS and 5 are wild type.

A similar experimental design with the same computational approach has been used to identify hits shared by multiple KRAS-dependent cell lines but not present in the wild type KRAS. In addition to the two AML cell lines, the spectrum of human cancer cell lines includes 12 cell lines from breast, colon, brain and ovarian and prostate cancers, 6 of which carry an activating KRAS mutation, and 6 expressing wild type KRAS.

The screen shows a tight correlation with KRAS dependency. The MDA-MB-231, DLD-1, RPMI-8226 and NOMO-1 cell lines all score as dependent on KRAS for their growth and survival. HCT-116 and the ovarian cancer cell line TOV21G do not score as KRAS-dependent even though they had previously been reported as being so.

After retesting, these two have been shown to represent false negatives, which is not entirely unexpected in this type of screen. The TOV21G cell line has mutated KRAS, but isn't dependent on KRAS for its growth and survival, at least as assessed by KRAS knockdown with shRNA.

Selective requirement

In heat maps of these results genes that score in mutant KRAS-dependent cell lines, but not wild type KRAS-expressing cell lines, KRAS itself scores the highest as a gene that is preferentially required by the mutant KRAS-dependent cells. The next highest gene on the map is STK33.

To validate the selective requirement for STK33 in mutant KRAS-dependent cells, a proliferation assay is used to analyze the effects of STK33 knockdown on cell viability in 7 AML cell lines. The cells are infected with a lentivirus containing a control shRNA, 2 shRNAs against KRAS and 2 shRNAs against STK33, selected with puromycin, and monitored over 4 days to generate growth curves.

In this assay, the shRNAs that target KRAS and STK33 have a strong anti-proliferative effect on mutant KRAS-harboring NOMO-1 cells, but not on THP-1 cells, which are mutated in NRAS.

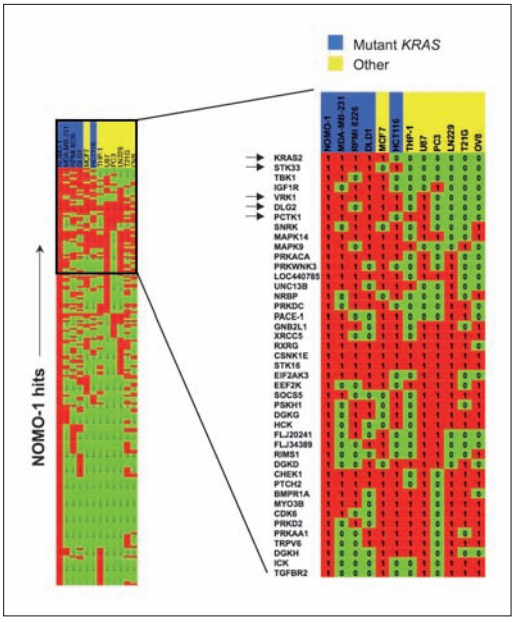


Figure 3
In a heat map of genes that score in mutant KRAS-dependent cell lines, STK33 is a top hit.

In this assay, the shRNAs that target KRAS and STK33 have a strong anti-proliferative effect on mutant KRAS-harboring NOMO-1 cells, but not on THP-1 cells, which are mutated in NRAS.

When the panel is expanded to include a spectrum of other hematopoietic cell lines that are known to be wild type for KRAS, there is no effect of knocking down either KRAS or STK33. Unexpectedly, however, knockdown of KRAS and STK33 inhibits the growth of two cell lines, NB4 and SKM-1, that have not been reported to harbor mutant KRAS.

To further assess this unanticipated result the KRAS coding region has been re-sequenced in NB4 and SKM-1s and has identified two non-canonical mutations, K117N and A18D, respectively. K117N had previously been reported in a colon cancer cell line but had not been functionally validated, and the A18D allele had not previously been described.

However, the KRAS 117N and A18D alleles, respectively, are fully transforming as assessed in anchorage-independent growth assays in NIH/3T3 cells *in vitro* and *in vivo*, in colony formation in semi-solid media, and confer factor independent growth to Ba/F3 cells. Thus, these functional assays indicate that the novel alleles are previously unidentified transforming alleles of KRAS.

A closer look at the consequences of KRAS and STK33 knockdown in the 7 AML cell lines thus indicates a relationship between KRAS dependency and STK33 dependency. Only mutant KRAS-dependent cell lines, and not cells expressing wild type KRAS, appear to be sensitive to STK33 inactivation.

This observation can be extended to other hematopoietic malignancy cell lines, including a multiple myeloma and a T-cell acute lymphoblastic leukemia. Each of these cell lines has mutant RAS, and is also sensitive to both KRAS and STK33 knockdown. In contrast, a multiple myeloma cell line that does not have mutant KRAS is unaffected by STK33 knockdown.

Codependent relationship

Interestingly, STK33 knockdown has no effect on one T-ALL cell line that carries a G12D KRAS mutation, but is not KRAS-dependent for proliferation *in vitro*. The results suggest that STK33 requirement is determined not by KRAS mutational status *per se*, but rather by dependence on KRAS for growth and survival.

Solid tumors might be expected to have some context-dependent differences in co-dependency on KRAS and STK33, but, in fact, they appear to behave in exactly the same way as cell lines derived from patients with hematologic malignancies. In solid tumor cell lines derived from different epithelial cancers, down regulation of STK33 inhibits the growth of those lines that are dependent on KRAS for growth and survival.

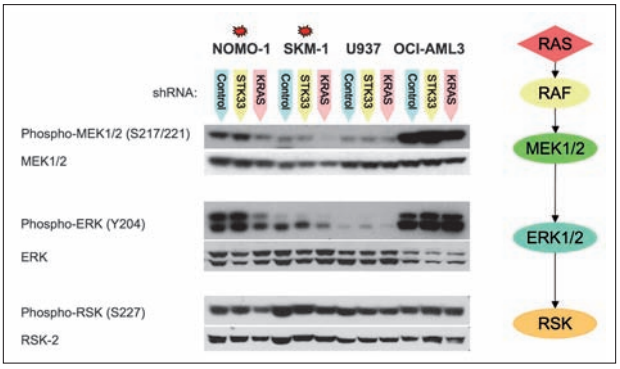


Figure 4
Effects of STK33 knockdown on signal transduction pathways.

STK33 appears to be down regulated by only about 50-60%, so these KRAS-dependent cell lines are exquisitely sensitive to STK33 knockdown, which may be useful from a therapeutic perspective.

When these same cell lines are stably transduced with a lentivirus expressing the hairpin of interest and implanted subcutaneously in a xenograft model, STK33 knockdown impairs growth of the xenograft, and markedly decreases their ability to form tumors.

In each case, a cell line that has wild type KRAS and is not dependent on KRAS for growth and survival is not sensitive to knockdown STK33. Taken together, these *in vitro* and *in vivo* data indicate that there is a functional relationship between dependency on KRAS and STK33.

Perhaps the most striking demonstration of this relationship comes from an experiment with HCT-15 and DLD-1 cell lines, which are sister cell lines derived from the same individual. Both lines have mutations in KRAS, but only DLD-1 is dependent on KRAS for survival and only DLD-1 is sensitive to STK33 knockdown. In contrast, HCT-15, which is not KRAS-dependent, is unaffected by STK33 knockdown. These data also corroborate with tumor growth in nude mice and in a xenograft model.

In a total of 26 cell lines representing 10 different tumor types, STK33 is required by cells that are dependent on KRAS for growth and survival, but not by cells that are wild type for KRAS. Remarkably, there is no divergence between KRAS and STK33 dependency.

These results suggest the possibility that small molecules targeting STK33 may provide a therapeutic window in a broad spectrum of human cancers associated with KRAS mutations.

Unknown entity

Little is known about STK33, or its biological function, making it difficult to determine the mechanism through which it might function in mutant KRAS-dependent cells.

The first paper about the gene, published in 2001, described its discovery, mapped its exon/intron structure and identified it as having homology with the calcium/calmodulin-dependent protein kinase subfamily[3]. However, STK33 itself doesn't have a calcium-binding motif, so it is almost certainly not a calcium-dependent kinase. The second paper, published in 2005, shows that STK33 is expressed in all tissues, but most highly expressed in breast, testes, lung and embryonic tissue, and is predominantly a cytoplasmic protein[4].

To explore the relationship between KRAS dependency and STK33, U937 and OC1-AML3 cell lines, neither of which are KRAS-dependent, are stably transfected with vectors containing KRAS G13D and a puromycin selectable marker. After selection with puromycin, the cell lines develop KRAS dependency as assessed by KRAS knockdown with shRNA. These cells, but not the parental wild type KRAS cells show concomitant sensitivity to STK33 knockdown, again providing a strong functional link between KRAS dependency and sensitivity to STK33.

To determine whether STK33 kinase activity is required for sensitization to STK33 in the context of KRAS dependency, a dominant-negative STK33 has been generated with a K145M substitution that renders the kinase dead. NOMO-1 and NB4 cell lines, which are both KRAS-dependent, grow well when stably transfected with wild type STK33, but transfection of the dominant negative STK33 confers lethality.

U937 cells, which express wild type KRAS and are not KRAS-dependent, are unaffected by the dominant negative STK33. These data are consistent with the idea that STK33 kinase activity is required for sensitization to STK33 knockdown in the context of KRAS dependency.

To assess consequences of STK33 knockdown on signal transduction pathways implicated in cancer, including the RAS/MAPK and PI3K/AKT pathways, western blot analysis has been performed using phosphorylation-specific antibodies to assess activation status of pathway intermediates.

There is no evidence in the RAS/MAPK pathways for a differential effect of STK33 knockdown in KRAS dependent versus the independent cell lines. What's more, knockdown of either KRAS or STK33 has no effect on the phosphorylation status of upstream components of the PI3K/AKT/mTOR pathway, regardless of KRAS status.

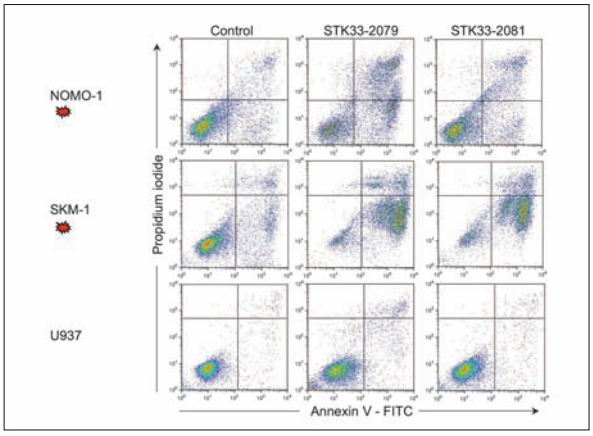


Figure 5
Genotype-selective induction of apoptosis by STK33 knockdown.

However, in KRAS-dependent cells, but not KRAS-independent cells, knockdown of either STK33 or KRAS markedly decreases the phosphorylation status of S6K1 that is a downstream effector of mTOR. The effect is particularly striking at S6K1 residue T389, which is known to correlate with the activation status of S6K1. This is of further interest because there is no detectable effect of the knockdown of STK33 on the upstream effectors of S6K1 mediated through the PI3K/mTOR pathway.

To verify that suppression of STK33 also reduces the enzymatic activity of S6K1, the phosphorylation status of two known S6K1 substrates, RPS6 and BAD, has been assessed. Knockdown of KRAS or STK33 also decreases the phosphorylation of RPS6 and BAD in KRAS-dependent cell lines, but not in KRAS-independent cells.

BAD protein

The pro-apoptotic protein BAD is normally phosphorylated and inactive. When it is dephosphorylated, it migrates to the mitochondria and induces mitochondrial-induced cell death. Consistent with this fact, knockdown of STK33 is associated with increased apoptosis in NOMO-1 and SKM-1 cells, which are dependent on KRAS for their growth and survival, but not in wild type KRAS-expressing U937 cells.

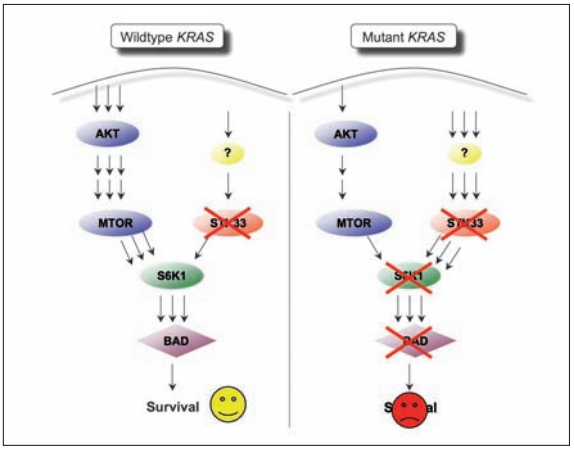


Figure 6
Model of STK33 dependency in cancer cells harboring mutant KRAS.

This effect should be specific for BAD if it is mediated by S6K1 phosphorylation of BAD, but not for other pro-apoptotic proteins such as BID that are not known to be regulated by S6K1. On consonance with this hypothesis, over expression of BAD, but not BID, rescues the cell death phenotype induced by STK33 knockdown specifically in KRAS dependent cell lines.

In summary, ectopic or endogenous expression of KRAS G13D induces KRAS and STK33 dependency. The catalytic activity of STK33 appears to be essential for the growth and survival of mutant KRAS-dependent cells. What's more, in the context of KRAS dependency, STK33 appears to regulate the phosphorylation and activity of S6K1, a major effector of mTOR, although SKT33 knockdown has no effect on upstream members of this pathway.

There are data suggesting that modulation of S6K1 by STK33 in the context of KRAS dependency occurs through an mTOR-independent mechanism. First, there is no evidence for a physical interactions between mTOR and STK33; S6K1 phosphorylation of BAD is thought to be regulated not by mTOR but by an as yet unidentified kinase; the effects of STK33 knockdown on cell viability and S6K1 activity cannot be rescued by knockdown of TSC2; treatment with rapamycin, a potent mTOR inhibitor, does not inhibit the growth of mutant KRAS-dependent AML cell lines; and finally, in NOMO-1 cells, knockdown of STK33 induces apoptotic cell death, but NOMO-1 cells over expressing wild type S6K1, or over expressing a mutated S6K1 that is mTOR-insensitive, are still sensitive to STK33 knockdown, as shown by the decrease in RPS6 phosphorylation.

One model for these results is that in cells that express wild type KRAS, dominant signaling occurs through the PI3K/AKT/mTOR axis to S6K1, and that results in BAD phosphorylation and survival of cells. However, as a consequence of the KRAS mutation, signal strength through this pathway is rendered weaker, such that S6K1 is more susceptible to regulation by STK33. Therapeutic targeting of STK33 may preferentially kill these mutant KRAS-dependent cells while sparing cells expressing wild type KRAS.

One interesting point is that the increased sensitivity of KRAS-dependent cells to STK33 does not appear to be related to the acquisition of mutations or amplification of STK33. There are no STK33 mutations in the coding sequence of any of the 26 cell lines, or in the hundreds of primary human cancer samples that are publicly available. There are also no differences in STK33 mRNA expression between cancers that have mutant or wild type KRAS.

In summary, there appears to be a synthetic lethal interaction between mutant KRAS dependency and suppression of STK33, and thus STK33 may be a potential target for the treatment of mutant KRAS-driven cancers with a broad therapeutic index. This will require the development of methods to identify oncogenic KRAS dependency in primary human tumors, to identify physiological roles for STK33, and to test the hypothesis with selective small molecule inhibitors of STK33.

Finally, these results indicate the potential for RNAi in discovering critical functional dependencies created by oncogenic mutations, and emphasize the complementarity between structural and functional cancer genomics.

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Drugging "undruggable" targets in cancer

A report on a lecture by
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One of the most vexing problems in life science is that of 'undruggability', the difficulty of targeting certain biological macromolecules in vivo using existing drug or ligand discovery technologies. As many as 80-90% of all potential targets, including many that have been extensively validated in humans and in animal models, are considered undruggable. Powerful new chemistry-based platform technologies may be able to convert these undruggable targets into legitimate targets for drug discovery. Specifically, there is great promise in synthetic biologics: molecules which, like biologics, possess the ability to target large flat surfaces, but which, like small molecules, are fully synthetic and hence can be modified at will. ***Gregory Verdine*** *presented progress on the development of synthetic biologics for so-called undruggable targets.*

The idea of druggable versus undruggable targets is often discussed in the research community. A retrospective look over the past 25 years reveals that, contrary to popular perception, most drugs that have been approved are not those based on synthetic molecules.

According to a 2007 compilation of all new classes of drugs including biologics and vaccines, about 5%, including rapamycin and cyclosporine, are natural products without any synthetic modification. Another 23% of drugs, such as rad001 and azithromycin, are natural products that have been synthetically modified.

There's one more class that is noteworthy: synthetic compounds that have natural product scaffolds — for example, a nucleoside analog that clearly traces its pedigree to natural products, but is fully synthetic.

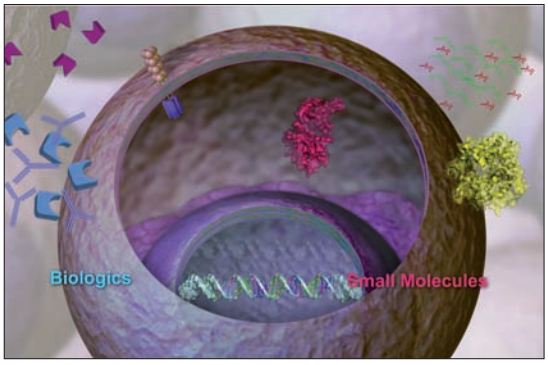

Figure 1
Classes of drugs and their targets.

The drug class that has been the subject of most investigation over the past 10-15 years is that of xenobiotics, which have no relationship in terms of their pedigree of structure to any naturally occurring compound. These drugs are fully synthetic, and their structure is designed based on other criteria, such as synthetic availability.

Altogether, 42% of drugs approved between 1981 and mid-2006 are related to natural products, while 40% have no natural product relationship in their structure — meaning that both categories have contributed equally over the past 25 years.

The decline in the pharmaceutical industry's investment in natural products is not matched by a decline in the rate of their approval, or in their overall contribution to approved drugs. By the same token, the expected increase in investment of xenobiotics has not resulted in a substantial increase in their approval.

These data make a strong argument that, as a class, xenobiotics are under-performing in terms of clinical approval, and natural products and natural product scaffolds are over-performing. Natural product compounds encompass all areas of therapy and, in fact, a much larger fraction have been approved as cancer drugs than as antimicrobials.

Synthetic biologics

There are multiple points of difference between the two categories of drugs. Natural products have lots of stereochemistry, a higher oxygen-to-carbon ratio, lower nitrogen ratio, and quaternary stereo centers, which are almost never seen in fully synthetic molecules. Although the complicated nature of the molecules makes it trickier to optimize them, it is arguably riskier to neglect them in favor of a drug class that is underperforming despite large investment.

In some companies, the Lipinski rules serve as rules or guidelines for how to develop orally active xenobiotics. Natural and semi-synthetic products do not conform to these rules. The fact that about half of the drugs approved over the past 25 years are in this class calls the Lipinski rules' validity into question.

The two major kinds of drugs are small molecules and biologics. Small molecules can almost always be synthetically modified to access targets inside and outside the cell. However, they have significant limitations in their targeting ability, and are restricted to targets that have a hydrophobic pocket on the surface.

Biologics, in contrast, are protein therapeutics. They have large contact surface areas, and they can target almost anything. But they're not able to get into cells so they are limited to targets that are outside the cell.

Extracellular targets account for about 10% of all targets in the human genome. Targets that have hydrophobic pockets comprise about another 10% of proteins coded by the human genome. So, the operating range of small molecules and biologics is tremendously limited, not being applicable to about 80% of targets.

This situation is what leads researchers to go after RAS farnesyl transferase instead of going after K-RAS itself or to try to find things downstream of c-MYC instead of targeting c-MYC itself — essentially pursuing targets that are not the ones most strongly indicated by the prevailing body of biologic data.

New classes of molecules that are larger than small molecules, with a larger contact surface area, can target things that small molecules cannot. But they still get into cells, so they can access intracellular targets.

Stapled peptides

Stapled peptides are a dominant negative strategy, in which the target is stripped down to its bare essence, to just the portion of the ligand that interacts with target.

Using the dominant negative peptide itself as the drug doesn't work because the rest of the protein provides the scaffolding for the peptide and forces it into its bioactive conformation. When the scaffolding is removed, the bioactive conformation of that peptide is lost.

Turning the peptide into a drug increases the problem exponentially because binding affinity is lost as a result of the unfolding, and the peptides are digested by proteases in a matter of seconds. With all the polar bonds exposed to the interior of the membrane, the peptides also create a high-energy barrier to transit across the membrane. Any one of these would be insurmountable problems but in combination, they have conspired to kill peptides as promising starting points for discovering drugs.

The simplifying notion in this is that all these problems derive from the unfolding of the peptide. Restoring the peptides' fold would make them bind more tightly, more proteolytically stable, and perhaps even help them get into cells.

One approach is to incorporate two non-natural amino acids into the peptide chain. These are simple relatives of alanine. They have an alpha-methyl group, as does alanine, but they also have a side chain with an olefin, which contains a carbon-carbon double bond. This allows the olefin ends to be crosslinked, thereby closing a large macrocyclic ring in the peptide – a hydrocarbon "staple."

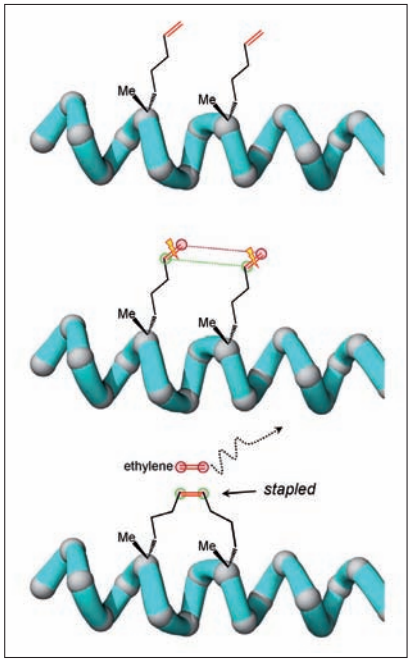

Figure 2
Peptide stapling uses a powerful new chemical reaction: olefin methasis.

The strategy combines two helix-stabilizing elements. The α-methyl groups program the peptide chain right at the site of incorporation to be an alpha helix, by exerting a local conformation effect. And the large macrocyclic ring has a global effect that restricts the number of things the peptide can do.

Introducing a hydrocarbon staple into the peptide makes it a more suitable candidate from a pharmacological standpoint. The chemistry is simple. The unnatural amino acids are incorporated at the *i* and *i*+4 position on the peptide, and lie on the same face of the helix. Alternatively, they can be located at the *i* and *i* +7 positions, which also works perfectly well[1].

The olefin metathesis breaks the olefinic bond in the non-natural amino acids, and reconnects the peptide-tethered carbons to form a new olefinic bond, which overall amounts to swapping the positions of the carbon-carbon double bonds (hence the name metathesis). Ethylene bubbles out of this reaction. Stapled peptides have been produced and purified on kilogram scale in preparation for the clinical testing of one of these stapled peptides, indicating that this is a manufacturable molecule.

Stabilizing effects

Cancer cells have various mechanisms to evade the apoptotic program. One of those mechanisms involves duplicating the gene encoding BCL2 family members, thereby creating a large reservoir for the effector proteins that ordinarily activate apoptosis.

The reading head of this apoptotic effector — its signature BH3 domain — can be embodied in a small stapled peptide and tested to see whether that stapled peptide can displace its parent protein once inside of cells, thereby reactivating the apoptotic response.

The peptides go from being about 15% helical when they are unstapled to about 88% helical when stapled. If the peptide is assessed more like a protein, the melting temperature of the unstapled peptide is below 15 degrees. Introducing just one staple into the peptide causes it to have a melting temperature of 64 degrees, a stability more similar to that of a thermophilic protein than of a small peptide. Thus, stapling the peptide essentially transforms it from something that has no stability as an alpha helix to something with a stability that's similar to that of a thermophilic protein.

Cellular uptake is generally the most vexing problem with peptides. In a fluorescence experiment, the stapled peptide shows clear fluorescence from inside cells, in contrast to the unstapled BH3 domain of the BID protein, which is not cell-permeable. Just putting the staple into the peptide allows it to get across cell membranes.

The stapled BH3 domain peptide gets across cell membranes via energy-dependent trafficking of endosomal vesicles. There is probably no specific receptor for this, but it may occur by an initial membrane association that requires the presence of the peptide. In the vast majority of cases, these vesicles uncoat in the 8-12

hour time range. The peptides are well below the exclusion size of the nuclear pore, so they may passively diffuse across the nuclear membrane and access nuclear targets.

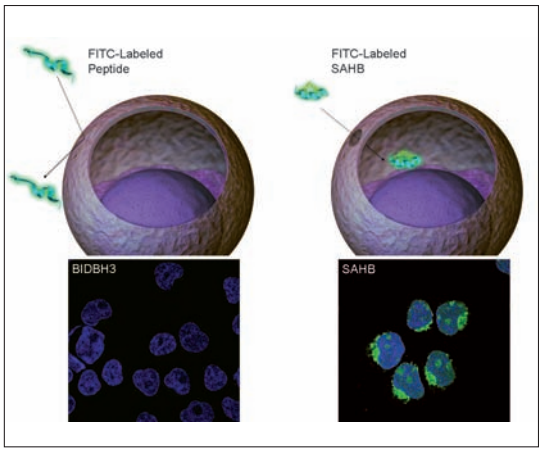

Figure 3
Intracellular uptake of SAHBA.

For standard mouse efficacy studies, refractory human leukemia cells are engineered to contain the gene encoding luciferase. Those cells are transplanted into mice and allowed to establish cells for 3 days, and then SAHBA, the stapled BID BH3 domain, is administered to the animals at 10 mgs/kg per day over 7 days. The tumor burden of the animals is then assessed by xenogen imaging.

By day 3, a very clear difference is observed between treatment with the stapled peptide and vehicle alone. That difference becomes more prominent after 5 and 7 days of the treatment cycle[2]. A control stapled peptide having a single point mutation that has a 10-fold reduced affinity for apoptotic targets exhibits substantially less efficacy in this mouse model.

Aileron Therapeutics has advanced this platform from a purely an academic effort, and selected the first clinical candidate based on the BIM BH3 domain. The BIM BH3s have shown widespread efficacy in liquid tumors and reasonable efficacy even in a range of solid tumors.

Peptide targets

The phosphorylated BAD protein is part of the glucose-sensing complex on the mitochondrial membrane. A phosphorylated version of the BAD stapled peptide has been shown to rescue the knockout of the BAD protein, acting as a gain-of-function effector[3].

There are also stapled peptides that potently inhibit HDM2 and HDMX, two important negative regulators of p53. Both these proteins bind the p53 activation domain, and that specificity is also embodied in the stapled version of the p53 activation domain.

An indium ligand is introduced onto the p53 stapled peptide, and indium-111 is loaded onto the conjugate. A soft tissue sarcoma is introduced into one footpad of a mouse, with the other, unaffected footpad being used as a negative control. The indium-labeled peptide is injected into the mouse and imaging conducted in the PET-MRI mode. The tumor-inoculated footpad clearly shows extensive uptake of the stapled peptide, indicating preferential uptake by the tumor; there is also uptake by the liver and to a lesser extent the kidneys, most likely reflecting routes of clearance[4].

This is evidence that transformed cells seem to turn over the membrane very quickly, and seem to be very effective at the kind of uptake mechanism that is being exploited. Stapled peptides can carry lots of other cargo including double-stranded RNAs, plasmids and proteins into cells, making this a reasonably effective way to engineer transport of heterologous molecules into cells.

Targeting Notch

One of the genes that's important during embryonic development and in some tissues post development is Notch. Notch is a transcriptional activator and it up regulates a whole series of genes, not the least of which is c-MYC, that is of interest to both pharmaceutical companies and to academics.

The structure of Notch provides a starting point for the development of stapled peptides. There is a DNA-

binding subunit, the subunit that is transported from the membrane to the nucleus upon cleavage by γ-secretase, a transcriptional co-activator, and a helix with two large segments.

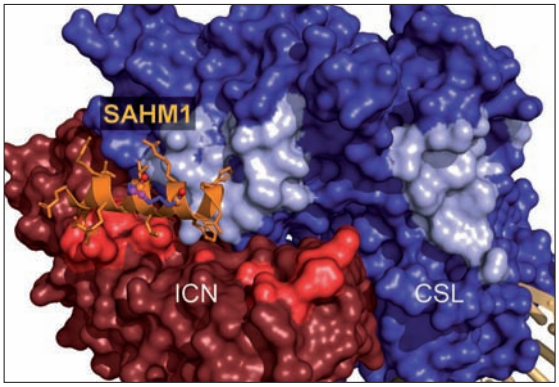

Figure 4
Stapled MAML peptides as "synthetic lethal" antagonists of NOTCH function.

In cell extracts and in cells, a stapled dominant negative peptide of MAML (SAHM1) pulls down the nuclear components of the Notch complex, which are known as CSL and ICN. When a double point mutation is introduced into this peptide, it is less effective at pulling down the components of the Notch complex.

The stapled peptide interferes with the ability of MAML to pull down the Notch complex, by interfering with its ability to associate with the nuclear components of the complex. Again, a single point mutation in this peptide abolishes its ability to pull down these components.

In KOP-TK1 cells, the stapled peptide can be shown to knock down canonical Notch-dependent genes, such as HES1, c-MYC and DTX1.

In the presence of a γ-secretase inhibitor, seven different T-ALL cell lines have been used to generate a set of 76 genes that are consistently and potently down regulated. KOP-TK1 cells treated with the stapled peptide SAHM1 generate a gene expression profile, and the genes most down regulated by either a γ-secretase inhibitor or a direct-acting NOTCH inhibitor are compared using a Gene Set Enrichment Analysis.

Looking at the genes whose expression is the most repressed or enhanced reveals that there is a tremendous clustering of the genes that are knocked down by the γ-secretase inhibitor and by a direct-acting transcriptional anatagonist of the Notch complex.

This is quite remarkable because if there were any significant levels of non-specificity with either the γ-secretase inhibitor or the stapled peptide, the gene sets would not be correlated. Instead, 9 of top 10 genes that are knocked down by the γ-secretase inhibitor and by the stapled peptide are the same. In contrast, there is a complete lack of correlation with the sonic Hedgehog gene set.

This stapled peptide kills cells that grow in a Notch-dependent way through apotosis, with kinetics that are considerably faster than that of a γ-secretase inhibitor. That may be because γ-secretase inhibitors work upstream, and there is a lot of Notch already present in the nucleus of those cells.

In a mouse model of T-ALL, driven by a constitutive Notch construct that has mutations in both the hetero-dimerization and the PEST domains, the mice develop a large population of CD8- and CD4-positive cells in the blood, bone marrow and spleen.

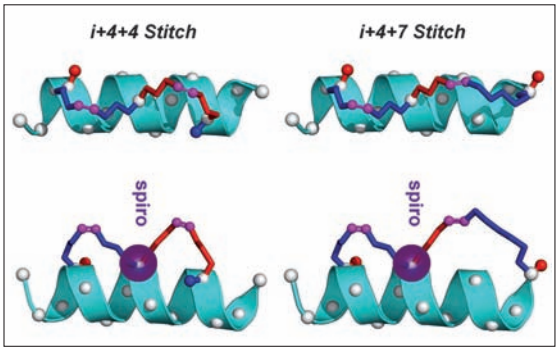

Figure 5
Two versions of stitched peptides.

When these animals are treated with the stapled peptide, there is a modest change in the tumor burden going from vehicle alone to an IP dose of SAHM1 of 35 mg/kg once a day. This becomes considerably more pronounced with twice-a-day IP dosing of the peptide at 30 mg/kg.

After 5 days of treatment, expression profiling of the blood of the treated animals showed a clear Notch inhibition signature, with a decrease in DTX1, c-MYC and HES1, the prototypical Notch-dependent genes.

Based on data in this and other cases, MYC, MITF and perhaps even HIF are likely to prove to be druggable using this kind of approach. The success with Notch is tantamount to a declaration of open hunting season on transcription factors.

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Abbreviations and glossary

1. ABBREVIATIONS

APL	acute promyelocytic leukemia
BCC	basal cell carcinoma
CDK	cyclin-dependent kinase
CML	chronic myelogenous leukemia
CTCL	cutaneous T-cell lymphoma
CTL	cytotoxic T lymphocytes
EGFR	epidermal growth factor receptor
GIST	gastrointestinal stromal tumors
GNMT	glycine N-methyl transferase
HDAC	histone deacetylase
HER2	human epidermal growth factor receptor 2
HSP-90	heat shock protein 90
IAP	inhibitor of apoptosis
LIC	leukemia-initiating cell
mTOR	mammalian target of rapamycin
NSCLC	non-small-cell lung cancer
PBMC	peripheral blood mononuclear cells
PET	positron emission tomography
PI3K	phosphoinositide 3-kinase
PSA	prostate-specific antigen test
RECIST	response evaluation criteria in solid tumors
SARDH	sarcosine dehydrogenase
TLR	Toll-like receptor
VELIPI	venous emboli in the tumor, lymphatic invasion and perineural infiltration

2. GLOSSARY

Terms in *italics* are defined elsewhere in the Glossary.

adaptive immunity also called acquired immunity; specific, targeted response to particular antigens, usually previously unseen molecules

adjuvant agents that modulate immune responses, often used to boost the immune response to vaccines

antigen a protein molecule recognised by the immune system; these may be proteins normally or abnormally present in or on cells, in which case they are termed self-antigens

apoptosis the orderly program of biochemical reactions leading to cell death and degradation

autophagy the process whereby the cell auto-digests itself to release nutrients and energy sources

chemokine *cytokines* that recruit or mobilize immune cells

cytokine molecules released by cells in response to infection or injury that stimulate inflammatory or healing responses

dendritic cells cells that initiate a primary immune response by activating lymphocytes and secreting cytokines

dimer protein molecule consisting of two subunits which may be the same (homologous) or different (heterologous)

domain region of a protein with a specific function, e.g., binding to receptor, *kinase* activity; sequence in a gene coding for such a region

inflammation response of the immune system to infection or irritation, characterized by redness and swelling

innate immunity pre-existing defenses that provide first-line protection against pathogens

kinase an enzyme that *phosphorylates* proteins, a part of the biochemical cascades involved in intracellular signaling

metabolism the set of chemical reactions that occur in living organisms in order to maintain life

metabolomics systematic study of the small-molecule profiles that are unique to various *metabolic* processes

metastasis the process of cells leaving the primary tumor and migrating through the lymph and blood systems to establish secondary tumors elsewhere in the body

monoclonal antibody antibody selected to recognize a specific sequence on the target protein or *antigen*

mutation a change in the DNA sequence of a gene that causes an alteration in the amino-acid sequence of the protein, often affecting its structure and/or function

MYC a transcription factor that regulates cell size, proliferation and differentiation, as well as sensitizing cells to apoptosis under stress conditions

oncogene a mutation in a gene involved in regulating cell proliferation or cell death that leads to neoplasia and maybe to malignant transformation

oncoprotein the protein product of an *oncogene*

phosphorylation the addition of PO_4- groups to a protein to activate or de-activate their interactions with other proteins in a *signaling cascade* (*see kinase*)

proteasome A large protein complex that degrades unneeded or damaged proteins

proto-oncogene a normal gene that can become an *oncogene* due to *mutations* or increased expression

RNA interference technique in which strands of double-stranded RNA are used to silence the expression of a gene

signal transduction any process by which a cell converts one kind of signal or stimulus into another, often involving ordered sequences of biochemical reactions.

SUMOylation post-translational modification of a protein that involves the attachment of Small Ubiquitin-like Modifier or SUMO

T cells white blood cells that play a central role in cell-mediated immunity

tyrosine kinase inhibitors drugs that block a tyrosine *kinase*, an enzyme that *phosphorylates* the amino acid tyrosine in nearby proteins to initiate a signaling cascade; tyrosine kinase inhibitors can prevent tumor growth, and may be used to treat cancer

ubiquitination post-translational modification of a protein that targets it for degradation by the attachment of ubiquitin, a highly-conserved regulatory protein

wild type gene of interest with no known *mutations*; animal carrying such a gene; often designated as +/+ if both alleles are wild type, or +/- if one allele is wild type, -/- indicates that both genes are mutated

xenografts human tumors grafted into immunocompromised mice

Kinase Drugs

Drug Name	Trade Name	Alternate Names	Target
Trastuzumab	Herceptin		HER2
Bevacizumab	Avastin		VEGFR
Cetuximab	Erbitux		EGFR
Panitumumab	Vectibix	ABX-EGF	EGFR
Ranibizumab	Lucentis		VEGFR
Dasatinib	Sprycel	BMS-354825	BCR/Abl, Src family, Eph family
Erlotinib	Tarceva	OSI-774	EGFR
Sorafenib	Nexavar		VEGFR
Sutinib	Sutent	SU11248	VEGFR
Imatinib	Gleevec, Glivec		Abl, kit
Nilotinib	Tasigna		BCR-Abl, Kit, PDGF
Temsirolimus	Torisel		mTOR
Gefitinib	Iressa		EGFR
Sirolimus	Rapamune	Rapamycin	mTor
Fasudil			ROCK1/2
Eupatilin			Erk1/2, CDKs?
Lapatinib	Tykerb		HER2/EGFR
Ruboxistaurin	Arxxant	LY333531	PKCb
Semaxanib		SU5416	VEGFR
CEP1347			Jnks
MGCD265			c-MET, VEGFR1, VEGFR2, VEGFR3, Tie-2 and Ron
Cediranib	Recentin	AZD2171	VEGFR
Alvocidib		Flavopiridol, HMR1275, L86-8275	CDK
Tozasertib		MK-0457, VX-680	Aurora kinase
R-roscovitine	Seliciclib	CYC202	CDK2/7/9
Axitinib		AG013736	VEGFR1-3, PDGF, Kit
Redeforolimus		AP23573, MK-8669	mTor
Vandetanib	Zactima	ZD6474	VEGFR, EGFR
Lestaurtinib		CEP-701	FLT3, JAK2
Everolimus	Certican, Afinitor	RAD-001	mTor
Bosutinib		SKI-606	Src
Neratinib		HKI-272	EGFR, HER2
Vatalanib		PTK787, PTK/ZK	VEGRF, PDGFR, KIT
		CEP-28122	ALK
		CEP-11981	VEGFR, Tie

Maker	Approval status	Chemical type
Genentech	Approved	Antibody
Genentech	Approved	Antibody
ImClone, Merck KGaA	Approved	Antibody
Amgen	Approved	Antibody
Genentech	Approved	Antibody fragment
Bristol-Myers Squibb	Approved for CML and Ph+ ALL	Small Molecule
Genentech/OSI	Approved	Small Molecule
Bayer/Onyx	Approved	Small Molecule
Pfizer	Approved	Small Molecule
Novartis	Approved	Small Molecule
Novartis	Approved	Small Molecule
Wyeth	Approved	Small Molecule
AstraZeneca	Approved	Small Molecule
Wyeth	Approved for renal cancer	Small Molecule
Schering	Approved in Japan	Small Molecule
	Approved in Korea only, for gastritis	Small Molecule
GlaxoSmithKline	Approved March 2007	Small Molecule
Eli Lilly	Approvable' pending additional trial	Small Molecule
Pharmacia	Failed phase III	Small Molecule
Cephalon	Parkinson's Trial Discontinued	Small Molecule
MethylGene	Phase I; Filing IND in 2007	Small Molecule
AstraZeneca	Phase I	Small Molecule
	Phase II	Small Molecule
	Phase II, enrollment halted in 2007	Small Molecule
Cyclacel	Phase IIa	Small Molecule
Pfizer	Phase III	Small Molecule
Merck, Ariad	Phase III (2008)	Small Molecule
AstraZeneca	Phase III (2008)	Small Molecule
Cephalon	Phase III for AML and Phase II for myeloproliferative disorders (2008)	Small Molecule
Novartis	Approved for kidney cancer	Small Molecule
Wyeth	Phase II	Small Molecule
	Phase II	Small Molecule
Bayer Schering, Novartis	Phase III (lacks efficacy?)	Small Molecule
Cephalon	Phase I	Small Molecule
Cephalon	Phase I	Small Molecule

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Participants in the group picture

Fondation Ipsen

The Fondation IPSEN, created in 1983 under the auspices of the *Fondation de France*, has two objectives: the distribution of knowledge and encouraging the exploration of emerging areas of research.

Contributing to the development and distribution of knowledge

One mission of the foundation is to promote interaction between researchers and clinicians by creating 'crossroads' and forums for fruitful exchanges. Today, with the extreme specialization of knowledge and the increasing mass of information that many find difficult to decipher, such exchanges are indispensable. For this to be effective, the foundation has focused on some of the crucial biomedical themes of our time: the spectacular developments in neuroscience and the scientific study of cognitive mechanisms, the challenges of neurodegenerative pathologies, the omnipresence of genetics and molecular biology, the growing field of endocrine interactions, and the problems of aging populations and theories of longevity. More recently, activities have expanded into two areas that are exciting for both their medical and fundamental challenges and that are currently in a phase of rapid development: cancer and the vascular tree.

Another goal of the Fondation IPSEN is to initiate, in partnership with the specialists and institutions involved, discussions and exchanges on the major scientific challenges of the future. Rather than trying to provide definitive knowledge, or to replace the work of large research organizations, the aim of these discussions is to emphasise multidisciplinary approaches at the boundaries of several disciplines, an approach that is essential for understanding the complexity and originality of human beings and their pathologies.

To fulfil these commitments, the foundation organises several series of international *Colloques Médecine et Recherche*, as well as funding awards to encourage research and publishing reports on its meetings. For each of these activities, the foundation brings together partners from the scientific and clinical world, who can independently report on the current state of knowledge and discuss the main issues in the areas on which the foundation has chosen to focus.

Over the past 25 years, the *Fondation IPSEN* has established its place in the scientific and medical landscape and intends to continue to be at the forefront in forming links, initiating multidisciplinary exchanges and contributing to the spread of knowledge, with time, intelligence, good will and above all, the collaboration of leaders in current biomedical research.

The Colloques Médecine et Recherche series

The *Colloques Médecine et Recherche* were created in 1987, with the first series dedicated to Alzheimer's disease. Its success stimulated the establishment of other several dedicated series: neurosciences, longevity, endocrinology, the vascular tree and more recently cancer. Meetings in each series are held annually, bringing leading international specialists together to present their most recent work, sometimes even before publication. Through these meetings, the *Fondation IPSEN* has over the years developed a large, international network of experts.

By focusing on emerging fields of knowledge, the meetings have supported the development of many new topics and have impacted on scientific advances in areas such as gene therapy and stem cells in the central nervous system, the role of cerebral amyloidosis in neurodegeneration, the contribution of genetic factors in resistance to disease, the benefits of neuronal grafts, biological markers of Alzheimer's disease, apolipoprotein E, brain-somatic cross-talk, relationships between brain and longevity, hormonal control of cell cycle to name a selection.

The series are organized around topics where active research is having or is likely to have a major impact on our knowledge:

• **Alzheimer's disease** – Since 1987, this topic has been explored at annual meetings that have followed or even anticipated the development of the new field of 'alzheimerology', which has gone beyond histology and neurochemistry to establish the underlying pathological mechanisms.

• **Neurosciences** – Started in 1990, this series of conferences has both enabled the identification of the major themes to emerge in this area and has supported not only the remarkable expansion of the neurosciences in the past fifteen years but also the effort to integrate its subdisciplines, from molecular mechanisms to human cognition.

• **Longevity** – Launched in 1996, this series examines the challenges and paradoxes of medicine by focusing on a positive aspect, cases of exceptional resistance to the effects of aging, rather than on disease. The evolution of research dedicated to *aging* into research dedicated to *longevity* represents a remarkable development in this field.

• **Endocrinology** – Established in 2002, this series examines the involvement of the endocrine system in the integration of all bodily functions. One example is the recent discovery of many hormones important in the control of metabolism, such as leptin and ghrelin. As aspects of brain-somatic crosstalk, such topics have impacts far beyond studies of hormones and the endocrine organs.

• **Vascular Tree** – This new series, begun in 2004, aims to examine the various steps that lead to development of the vascular system, its growth in harmony with that of other organs, its degeneration, death and the possibilities for its regeneration. A new vision is emerging of blood vessels not as simple 'pipes' but as living, complex organs with interactions throughout the body.

• **Cancer Science** – Three annual experts meetings have been organized in collaboration with Inder Verma and the participation of remarkable leading opinion makers in the field. Challenging topics (Can Cancer be Treated as a Chronic Disease?, Are inflammation and Cancer Linked?, Metastasis and Invasion, Metabolism and Cancer, Molecular Targets of Cancer Therapy) have generated outstanding discussions among the participants. The 2010 meeting will deal with the link between stem cells and cancer.

Partnerships

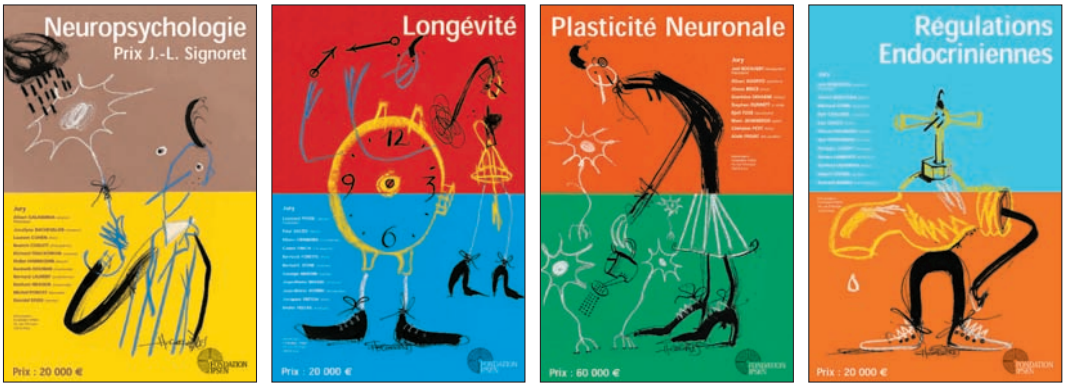
Long ago, the *Fondation IPSEN* has developed partnerships with international institutions and organisations, to encourage cooperation between experts in various disciplines. These partners include: the World Health Organisation (WHO), the *Fondation Nationale de Gérontologie* (FNG) and Harvard University.

Three additional partnerships were implemented in 2007:

- First with the **Salk Institute** (La Jolla) and **Nature Publishing Group**. This partnership consists of a series of annual meetings on "Biological Complexity": *Transcription diseases (2007), Genes, Circuits and Behavior (2008), Processes of Aging (2009), Sensory Systems : Small Taste, Touch, Hearing and Vision (2010).*
- Second with **Cell Press** and the **Massachusetts General Hospital**. This series, "Exciting Biologies", has been initiated in 2007, with a meeting entitled *Biology in Motion*. The next meetings were: *Biology of Cognition (2008), Biology in Balance (2009).*
- And finally with **Nature Publishing Group** on the general theme of "Emergence and Convergence", four meetings a year are being organized in various domains. The New York Academy of Sciences hosted the first of the series in October 2007, *Small RNAs in Development, Immunology and Cancer*. Since then, five other meetings have been held: *Genome Evolution and Structural Variation (Seattle), Epigenetics and Behavior (Houston), Multiple Sclerosis: From Pathogenesis to Therapy (Paris), Cell shape and Polarity (Chocago), Mitochondrial Dysfunction in Neurological Disease (Durham).*

Awards to Encourage Research

The *Fondation IPSEN* awards prizes to researchers who publish remarkable, pioneering studies. Currently, four awards are given annually:



Posters advertising the Fondation Ipsen prizes.

• **The Neuronal Plasticity Award** has been given each year since 1990 to three researchers working on the same theme: Albert Aguayo, Anders Björklund and Fred Gage; Ursula Bellugi, Wolf Singer and Torsten Wiesel; Philippe Ascher, Kjell Fuxe and Terje Lomo; Per Andersen, Masao Ito and Constantino Sotelo; Mariano Barbacid, Yves Barde and Hans Thoenen; Jacques Melher, Brenda Milner and Mortimer Mishkin; Friedrich Bonhoeffer, Cory Goodman and Marc Tessier-Lavigne; Antonio Damasio, Richard Frackowiak and Michael Merzenich; Heinrich Betz, Gerald Fischbach and Uel McMahan; Masakazu Konishi, Peter Marler and Fernando Nottebohm; Tomas Hökfelt, Lars Olson and Lars Terenius; Albert Galaburda, John Morton and Elizabeth Spelke; Arturo Alvarez-Buylla, Ron McKay and Sam Weiss; François Clarac, Sten Grillner and Serge Rossignol; James Gusella, Jean-Louis Mandel and Huda Zoghbi; Ann Graybiel, Trevor Robbins and Wolfram Schultz; Mary Kennedy, Morgan Sheng and Eckart Gundelfinger; Nikos Logothetis, Keiji Tanaka and Giacomo Rizzolatti; Jean-Pierre Changeux, Peter Kalivas and Eric Nestler; Alim-Louis Benabid, Apostolos P. Georgopoulos, Miguel A. L. Nicolelis.

• **The Endocrinology Award**, first given in 2002, has been received by Wylie Vale, Robert Lefkowitz, Pierre Chambon, Tomas Hökfelt, Roger Cone, William Crowley, Ron Evans and Gilbert Vassart.

• **The Jean-Louis Signoret Neuropsychology Award**: since 1992, the recipients have been Eric Kandel, Jacques Paillard, Rodolfo Llinas, Steven Kosslyn, Alfonso Caramazza, Jean-Pierre Changeux, Emilio Bisiach, Joseph LeDoux, Joaquim Fuster, Stanislas Dehaene, Deepak Pandya, Utah Frith, Antonio and Hanna Damasio, Marc Jeannerod, Faraneh Vargha-Khadem, Alvaro Pascual-Leone, Elizabeth Warrington and Pierre Maquet.

• **The Award for Longevity**, created in 1996, has been bestowed on: Caleb Finch, Vainno Kannisto, Roy L. Walford, John Morley, Paul and Margret Baltes, Justin Congdon, George Martin, James Vaupel, Linda Partridge, Sir Michael Marmot, Cynthia Kenyon, David Barker, Gerald McClearn and Jacques Vallin.

International Publications

Books summarizing of the conferences organised by the Fondation IPSEN are published in English and distributed by international publishers:

• Research and Perspectives in Alzheimer's Disease (Springer, 24 titles)

• Research and Perspectives in Neurosciences (Springer, 17 titles)

• Research and Perspectives in Longevity (Springer, 5 titles)

• Research and Perspectives in Endocrinology (Springer, 8 titles)

• WHO/Ipsen Foundation series (Springer, 7 titles)

• Brain and Mind Collection

Books and brochures recently published by the *Fondation Ipsen.*

In addition, since 1986 the Fondation IPSEN has published more than 200 issues of *Alzheimer Actualités*, a newsletter dedicated to Alzheimer's disease; in 1993, a bi-annual journal, the *Bulletin du Cercle de Neurologie Comportementale* was started; and in 2005, the first of two series of annual reports on the conference dedicated to *Cancer Science* and the *Vascular Tree* appeared. The foundation also has widely distributed information in various forms to the medical professions and families of patients, as well as produced teaching films that have received awards from specialized festivals.

FONDATION IPSEN

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