The history of the kinome and how this has facilitated development of kinase inhibitors

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Protein kinases and phosphatases are key components of protein phosphorylation based signaling networks in eukaryotic cells. In consequence, there has been long-standing interest in the number of protein kinases and phosphatases needed to constitute intracellular signaling networks. We have used complete genome sequences combined with cDNA and EST sequence information to determine the total number of protein kinase genes (the kinome) in budding yeast, C. elegans, Drosophila, mouse, and Homo sapiens (mouse is ~99% identical and chimpanzee is essentially identical). The human kinome has 518 PK genes; 478 of these PKs belong to the eukaryotic protein kinase (ePK) superfamily, and the remainder are atypical protein kinases (aPK), divided among a few small families. Over 50 of the ePK catalytic domains in the human kinome are missing one of more of the key ePK catalytic residues, and are therefore predicted to lack catalytic activity; these domains presumably have functions other than phosphate transfer. Comparison of the kinomes from different eukaryotic species provides insights into the evolutionary history of the protein kinases. Protein kinases constitute ~2% of all genes in budding and fission yeast, worms, flies and humans, and ~4% in plants. In metazoans 15-20% of all protein kinases are tyrosine kinases, but true tyrosine kinases are lacking in protozoans, such as the yeasts. The existence of tyrosine kinases in simple metazoans suggests that tyrosine phosphorylation evolved as a mechanism for intercellular communication. Analysis of more recently completed kinomes, including Tetrahymena and Dictysostelium, shows that the tyrosine kinase-like kinases (TKLs) evolved in unicellular organisms, perhaps serving as the antecedents to the true tyrosine kinase, and were secondarily lost from the yeasts. Two-component “histidine” kinases, which are quite common in some protozoans and plants, were lost during the evolution of metazoans. The kinome was already well diversified in early deuterostomes; for instance, the sea urchin kinome lacks only 4/187 human protein kinase families.

The already significant complexity of the kinome at the gene level is increased by the use of alternative promoters and terminators, and by alternative splicing, which results in an average of ~3.5 distinct coding products per kinase gene. Consistent with the large number of distinct protein kinase entities expressed in a single cell, recent MS-based phosphoproteomic analyses indicate that a very large proportion of intracellular proteins is phosphorylated, often at more than one site, and that a significant fraction of all of the many thousands of phosphorylations in a cell can change in response to external stimuli.

Mutations in protein kinase and phosphatase genes are increasingly found to be causal in human diseases; out of 518 protein kinase genes >150 have been implicated in disease. Activating or inactivating mutations, overexpression or underexpression of >120 tyrosine and serine kinases has been associated with human cancer. Genomic sequencing of protein kinase and phosphatase exons and regulatory regions for activating/inactivating mutations in cancer has yielded many additional cancer-causing protein kinase and phosphatase gene candidates. The prevalence of protein kinases involved in disease has led to intensive efforts to develop specific protein kinase inhibitors, and major efforts are underway in the pharmaceutical industry and academia to develop protein kinase inhibitors as cancer therapeutics and treatments for other diseases. Five tyrosine kinase inhibitors (TKIs) (Gleevec, Iressa, Tarceva, Sutent, Sprycel) and one serine/threonine/tyrosine kinase inhibitors (Nexavar) are approved for clinical use in cancer therapy in the US; >70 other protein kinase inhibitors are in cancer clinical trials, and >25 protein kinase inhibitors are in trials for diseases other than cancer. In addition, rapamycin analogues, which inhibit the mTOR aPK, are also in clinical trials for several cancers. One can safely predict that in the next decade increasing numbers of protein kinase (and phosphatase)-targeted drugs will become available for use in treatment of cancer and other human diseases, both singly and in combination.

Session I - 2

Regulation of kinase signaling by protein interaction domains

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A primary mechanism for signaling by receptor tyrosine kinases (RTK) involves autophosphorylation, followed by the recruitment of cytoplasmic targets with SH2 or PTB domains, which directly recognize phosphotyrosine sites on activated receptors. SH2-containing proteins subsequently stimulate intracellular signaling pathways, or regulate receptor kinase activity. Autophosphorylation within the kinase or juxtamembrane regions of RTKs also has a direct influence on the conformation and activity of the catalytic domain. For example, Eph receptors are primarily activated by autophosphorylation in the juxtamembrane region. Using a combination of x-ray crystallography, NMR spectroscopy and mutagenesis we have shown that autoinhibiton by the juxtamembrane region restricts the dynamic movements of the kinase, and that this is relieved upon autophosphorylation.

In contrast to RTKs, cytoplasmic tyrosine kinases possess intrinsic interactions domains (i.e. SH2, SH3, PH, FERM), which can both regulate kinase activity through intramolecular interactions, and direct the activated kinase to specific substrates once this inhibition has been relieved. Such interactions can be exploited by kinase inhibitors.

Based on this model, we have surveyed the kinome for non-catalytic interaction domains, and find by hierarchical clustering that protein kinases fall into groups, based on the identities of their interaction domains, which closely reflect the organization of the kinome derived from sequence analysis of the kinase domains. These results suggest a close regulatory interplay between kinase and interaction domains. Indeed, there is a large and growing number of interaction domains that recognized serine/threonine phosphorylated sites, in a similar fashion to the binding of SH2 domains to phosphotyrosine sites. Proteomic analysis of such phospho-dependent interactions will be discussed.

Oncogenome analysis as basis for cancer drug development

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Cancer represents a disease prototype that is connected to defects in the cellular signaling network that controls proliferation, motility, survival and recognition by the immune surveillance. The first insights into the genetic basis of cancer were obtained by comparing the sequences of retroviral oncogenes with human proto-oncogenens. Currently the spectrum of known genetic alterations in cancer cells includes mutations in various genes leading to structural and functional dysfunctions in signal transmission as well as over- or under-expression of positive or negative signal regulatory proteins respectively. For the past years we have investigated various aspects of signaling systems in tumor cells in order to identify critical switch points in the pathophysiological process that results in malignancy. These efforts aim at the selective blockade of abnormal, disease-promoting signaling mechanisms by monoclonal antibodies, or small molecule kinase inhibitors rather than the eradication of all growing cells in the body as in the case of most currently used chemotherapeutic drugs. This strategic approach began with the cloning of the EGF receptor cDNA and the related receptor HER-2/neu and translated the animal oncogene concept into target-directed therapy of human cancer. This work yielded the first specific oncogene target-based, FDA-approved (1998) therapeutic agent, “Herceptin”, for the treatment of metastatic breast cancer that is charachterized by HER2 overexpression. Subsequent “target-driven drug development” efforts that employed various genomic analysis strategies led to the identification of the receptor tyrosine kinases FGFR4, Axl/Ufo and Flk-1/VEGFR2 as critical signaling elements in tumor progression. The latter served, in cooperation with SUGEN Inc./Pharmacia/Pfizer, as basis for the development of anti-angiogenic small molecule drugs SU5416, SU6668 and SU11248. The drug discovery process that led to SU11248 represents a prototypical example for the adaptation of cancer therapeutics from highly specific to multi-targeted drugs. In January 2006 the FDA approved SU11248/SUTENT for the treatment of Gleevec-resistant GIST and Renal Cell Carcinoma (Pfizer) and on July 19 the European Agency EMEA followed suit. New developments and insights that were gained over the past twenty years of targeted cancer therapy research and development will be discussed.

Cell signaling by receptor tyrosine kinases

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Receptor tyrosine kinases (RTKs) comprise a large family of cell surface receptors that control many critical cellular processes. The intrinsic protein kinase activity of RTKs is stimulated following growth factor binding to the extracellular ligand-binding domain which stimulates receptor dimerization, tyrosine autophosphorylation and enhancement of enzymatic activity leading to the recruitment and activation of multiple intracellular signaling pathways. It is now well established that various human diseases and pathologies are caused by dysfunction in RTKs or in the intracellular signaling pathways that they activate. These include many cancers, developmental abnormalities, sever bone disorders, immune diseases, arteriosclerosis and angiogenesis among others.

We have used mass spectrometry and X-ray crystallography to demonstrate that tyrosine autophosphorylation of the catalytic tyrosine kinase domain of FGF-receptor-1 (FGFR1) is mediated by a sequential and precisely ordered reaction. We also demonstrate that the rate of catalysis of two FGFR substrates is enhanced by 50 to 100 fold following autophosphorylation of the first site in the activation loop while autophosphorylation of the second site in the activation loop results in 500 to 1000 fold increase in the rate of substrate phosphorylation. We propose that FGFR1 is activated by a two-step mechanism mediated by strictly ordered and regulated autophosphorylation suggesting that distinct phosphorylation states may provide both temporal and spatial resolution to receptor signaling. Genetic models in mice provide new opportunities for exploring and developing new treatments for diseases caused by dysfunctions in RTKs and in their intracellular signaling pathways. Inhibitors of tyrosine kinases have been successfully applied for the treatment of cancers driven by activated tyrosine kinases. Sutent/SU11248 is a new drug that blocks the actions of several tyrosine kinases including c-Kit, PDGFR and VEGFR. Sutent has been approved by the FDA for the treatment of gastrointestinal stromal tumors (GIST), Gleevec resistant GIST, and for advanced kidney cancers. The approval marks the first time the FDA has approved a new oncology product for two indications simultaneously.

The ERBB family of receptor tyrosine kinases and cancer

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The ErbB receptors (EGFR, ErbB2, ErbB3 and ErbB4) have essential roles in normal physiology and EGFR and ErbB2 in particular are involved in the development of numerous types of human tumors. Cancer development is a multistep process starting from a local benign hyperplasia and ending with an invasive tumor able to metastasize to distant organs. ErbB receptors and their ligands have been shown to play roles in most, if not all of the steps. Accordingly, EGFR and ErbB2 have been intensely studied both to understand their roles in tumor cell biology and to optimize their usefulness as therapeutic targets. Three major mechanisms contribute to aberrant EGFR and ErbB2 receptor activity in cancer: gene amplification leading to receptor overexpression, activating mutations in the kinase domain of EGFR and to a lesser extent ErbB2, and autocrine ligand production causing constitutive receptor activity. During the presentation different aspects related to in vitro as well as in vivo response of tumor cells to ErbB targeted inhibitors will be discussed and new insight into resistance mechanisms will be mentioned. Finally, experiments on a novel ErbB receptor effector protein and its potential to serve as a point of intervention in the malignant process will be presented.

Targeting the VEGFR-3 pathway for inhibition of lymphangiogenesis and metastasis

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Angiogenesis and permeability of blood vessels are regulated by vascular endothelial growth factor (VEGF) via its two receptors VEGFR-1 and VEGFR-2. The VEGFR-3 receptor does not bind VEGF and its expression becomes restricted mainly to lymphatic endothelia during development. We have found that homozygous VEGFR-3 targeted mice die around midgestation due to failure of cardiovascular development. We have also purified and cloned the VEGFR-3 ligand, VEGF-C. Transgenic mice expressing VEGF-C show evidence of lymphangiogenesis and VEGF-C knockout mice have defective lymphatic vessels. The proteolytically processed form of VEGF-C binds also to VEGFR-2 and is angiogenic. VEGF-D is closely related to VEGF-C, similarly processed and binds to the same receptors. Thus VEGF-C and VEGF-D appear to be both angiogenic and lymphangiogenic growth factors. VEGF-C overexpression led to lymphangiogenesis and growth of the draining lymphatic vessels, intralymphatic tumor growth and lymph node metastasis in several tumor models. Furthermore, soluble VEGFR-3, which blocked embryonic lymphangiogenesis, also blocked lymphatic metastasis in breast and lung cancer models. These results together with recent clinical cancer studies suggest that paracrine signal transduction between tumor cells and the lymphatic endothelium may be involved in lymphatic metastasis of human cancers.

1. *Alitalo et al. Lymphangiogenesis in development and human disease. Nature 438 (7070): 946-53, 2005.*
2. *Tammela et al. Molecular lymphangiogenesis: new players. Trends Cell Biol 15: 434-41, 2005.*

Signaling via receptors for PDGF and TGF-beta - possible targets for tumor therapy

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Platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-beta) affect cell growth, survival and migration, and have important functions during the embryonal development.

PDGF isoforms exert their cellular effects via two structurally similar tyrosine kinase receptors. Since PDGF promotes cell growth and survival, overactivity of the PDGF signaling pathway is associated with diesease, e.g. malignacies. We have explored the use of PDGF antagonists in tumor treatment, and found efficient inhibition of tumor growth in animal models of tumors driven by autocrine PDGF production. In addition, we have observed that inhibition of paracrine PDGF stimulation of stromal fibroblasts and vessel pericytes lowers tumor interstitial fluid pressure and tumor angiogenesis.

TGF-beta has a more complicated role in cancer; initially TGF-beta is a tumor suppressor through its ability to inhibit growth and to promote apoptosis of tumor cells. At later stages, when tumor cells become insensitive to the cytostatic effects of TGF-beta, TGF-beta has tumor promoter effects through stimulation of epithelial-to-mesenchymal transition of tumor cells, stimulation of angiogenesis and suppression of the immune system. We are currently delineating the signaling pathways involved in the various cellular effects of TGF-beta, and exploring the possible use of TGF-beta antagonists in tumor treatment.

Tageting protein kinases for the development of anti-inflammatory drugs

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Infection by pathogens, such as bacteria and viruses, triggers macrophages to produce pro-inflammatory cytokines (PIC) which mount the innate immune responses to fight the invading organism. However, this defence mechanism is a double-edged sword, because the uncontrolled production of PIC is a major cause of chronic inflammatory diseases, such as rheumatoid arthritis, as well as septic shock. For this reason many pharmaceutical companies are trying are trying to develop anti-inflammatory drugs that suppress the production of PIC.

In this talk I will present some of our recent research aimed at dissecting the signalling pathways that trigger the production of PIC in response to pathogens. In particular, I will discuss our progress in understanding the roles of the protein kinases IRAK1 (interleukin-1 receptor associated kinase 1), RIP2 (receptor-interacting kinase 2) and COT/Tpl2, as well as the role of Lys63-linked polyubiquitination in this process.

Session IV - 1

From Gleevec to targeting the kinome

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Imatinib (Gleevec; STI 571; CGP 57148) exemplifies a molecularly targeted drug that has revolutionized the therapy of chronic myeloid leukemia (CML). The clinical success of Imatinib has stimulated major interest in the kinases field and led to the hope that this development paradigm would translate to other malignant and non-malignant diseases.

A number of kinase inhibitors are now on the market and their application in clinical medicine is being elucidated both rationally and empirically. Additionally, many inhibitors are currently entering or progressing through clinical trail. Many of these inhibitors target a small group of ‘validated” targets, with specificities ranging from narrow to extremely broad. The true kinome inhibition profile of many of these inhibitors and their related analogues are not known, obscuring the mechanistic interpretation of efficacy and toxicity findings. On the other hand, target promiscuity within chemical series can be an advantage when searching for leads against novel targets within the kinome. Selective issue in the discovery and development of Imatinib will be discussed and contracted to current approaches to kinome drug discovery.

Nilotinib: a new agent for the treatment of imatinib-resistant CML

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Imatinib (Glivec®), an inhibitor of the tyrosine kinase activity of the Bcr-Abl oncoprotein, is an effective therapy of chronic myelogenous leukemia (CML). However, patients with advanced stage disease frequently relapse due to the emergence of imatinib-resistant mutants of Bcr-Abl. Consequently, a potent Bcr-Abl inhibitor, which maintains activity against imatinib-resistant mutants, should provide patient benefit in CML. Based upon our x-ray crystallographic analysis of the binding mode of imatinib [1], we prepared libraries of compounds designed to probe the inactive conformation of the human Bcr-Abl kinase domain [2], and identified some key pharmacophore elements. Optimisation of the biopharmaceutical properties of lead compounds, afforded potent, selective Bcr-Abl kinase inhibitors, possessing good pharmacokinetic profiles and showing efficacy following oral administration in murine models of CML [3]. Nilotinib (Tasigna®), which has recently completed Phase II clinical trials in imatinib-intolerant and imatinib-resistant CML patients, emerged from these studies [4].

[1] Cowan-Jacob SW, Guez V, Griffin JD, et al. Mini Rev. Med. Chem. 2004;4:285.
[2] Manley PW, Breitenstein W, Brüggen J, et al. Bioorg. Med. Chem. Lett. 2004;14:5793.
[3] Weisberg E, Manley PW, Breitenstein W, et al. Cancer Cell 2005;7: 129.
[4] Kantarjian H, Giles F, Wunderle L, et al. New Engl. J. Med. 2006;354:2542.

Kinases of mycobacteria and plasmodia as potential drug targets

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Over the last 20 years, an enormous expertise has been assembled in targeting kinases for cancer therapy, using the most advanced biomedical technologies on a broad front, and this effort has resulted in several successful drugs. Neglected diseases including tuberculosis (TB) and malaria have not benefited from these concepts and resources, and it is legitimate to ask whether such an expertise could be usefully employed in the framework of these and related diseases.

Drug targets must generally fulfill base-line requirements including essentiality (is the microbe dependent on the function of the drug target, at least some of the time, at relevant growth conditions), epidemiology (is the target present in clinically relevant strains) and drugability (how easy is it to find pharmacologically meaningful leads).

A genomic analysis of the clinically most relevant strain H37Rv of the prokaryotic *Mycobacterium tuberculosis* (Mtb), using different resources including HAMAP and Swiss Prot, has identified 75 unique proteins of which there are 11 putative serine / threonine kinases (Pkn), and 12 putative two component sensors (transmembrane histidine protein kinases). For instance, PknA and PknB seem to be involved in cell wall synthesis and shape control, while PknF and PknG may be involved in the pathogenesis of Mtb. Additionally, Mtb uses various kinases in metabolic pathways such as aroK (shikimate kinase) in the folate and other pathways and CoaA (pantothenate kinase) which is involved in the activity of CoA (Coenzyme A) and (ACP) Acyl Carrier Protein and is a vitamin (B5) in the mammalian host. These pathways are unique for the microbial cells and offer therefore potentially selective drug targets that leave host function unaffected.

Many different protein kinases can be found in eukaryotic plasmodia such as *Plasmodium falciparum* (Pf) and *Plasmodium vivax* (Pv), that are two important causative agents of clinically distinct forms of malaria. The genomes of both parasites have been fully sequenced, and PlasmoDB is the essential resource. C. Doerig's group (BMC Genomics, 2004, **5**:79) has analyzed the kinase families of Pf identifying 65 eukaryotic protein kinases that can be grouped into several subfamilies: the casein kinase group (1 member), the AGC group

(cAMP-, cGMP-dependent kinases, protein kinase C, 5 members), the CMGC group (cyclin-dependent kinases, mitogen-activated protein kinases, glycogen synthase kinase, 18 members) and several other groups.

The structural divergence between the malarial protein kinases characterized to date and their mammalian homologues supports the idea that specific inhibition of parasitic enzymes can be achieved (Keenan, S.M. and W.J. Welsh, J Mol Graph Model, 2004. **22**(3): p. 241-7, Holton, S., et al., Structure (Camb), 2003. **11**(11): p. 1329-37. Indeed, species-dependent differential susceptibility of orthologues of some PK classes to a given compound has been described. Together with the high probability that many of these enzymes are essential to parasite survival, this makes protein kinases attractive antimalarial drug targets.

Session V - 1

Treating the kinase signaling addiction in solid tumors: lessons learned from GIST as a paradigm of molecularly targeted therapy

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Gastrointestinal stromal tumor (GIST), a form of soft-tissue sarcoma, is the most common noncarcinomatous tumor of the gastrointestinal tract. Despite its high incidence of recurrence, the malignant potential of GIST has been under-recognized. Advances in diagnostic technology since 2000 have led to increased diagnoses of GIST, suggesting that GIST is more common than previously suspected. Historically, the only treatment for GIST was surgical resection, but recent advances in the understanding of the pathogenesis of the disease have led to the development of a new treatment. A key factor in the growth and survival of cancerous GIST cells is the uncontrolled activation of a signaling enzyme known as KIT, a receptor tyrosine kinase, which becomes locked in an activated state. The abnormal signaling from the overactive KIT enzyme causes GIST cells to survive and proliferate uncontrollably. Imatinib mesylate is an oral drug designed to inhibit the kinase enzyme activity of KIT. Imatinib has been proven in several clinical trials to be effective against GIST and is currently the firstline medical therapy for malignant metastatic or recurrent GIST. Imatinib is administered as an outpatient oral drug and warrants nursing management with particular attention to potential side effects, significant drug interactions, monitoring, and patient education. This article--based on published trials and clinical experience--summarizes the nursing implications, clinical efficacy, and safety of imatinib as an effective and rationally targeted treatment for GIST.

Targeting IGF1R, PKB/Akt and EGF receptor

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In this discussion we shall report on novel, high affinity substrate competitive inhibitors, which target IGFR (1) and PKB/Akt (2,3). The agents posses strong antiproliferative/pro-apoptotic activities on breast, prostate and pancreatic cancer cell lines in tissue culture and in xenograft mouse models. We also show that their toxicity and stability profile make them promising candidate agents against a wide spectrum of cancers. I shall discuss the overall advantage of substrate-mimics vis-à-vis ATP-mimics as kinase inhibitors, especially when one deals with Serine kinases. Since EGF receptor kinase inhibitors (tyrphostins) perform weakly in the clinic, where Erbitux is doing slightly better, we developed a new approach to treat tumors that over-express the EGFR. The approach is not based on the inhibition of EGFR but rather on the selective internalization of PolyIC (synthetic long dsRNA) by a chemically defined vector homing to the EGF receptor. This strategy brings about the total eradication of EGFR over-expressing glioblastoma (4) as well as EGFR over-expressing disseminated tumors (5) in experimental animals. This strategy is applicable to every tumor that over-expresses EGFR, independently of the role the receptor plays in the biology of the tumor. Since a significant fraction of all human cancers over-express EGFR, the newly developed “Trojan horse” can be utilized in a significant fraction of lung cancers, head and neck cancer, breast cancer and more. Since the structure of the chemical vector is modular, where its “head” is the growth factor, it can be adapted to target any receptor that is over-expressed on the tumor cell and undergoes ligand induced receptor internalization.

1. *ATP non-competitive IGF1 receptor kinase inhibitors as lead anti-apoptotic and anti-Papilloma agents. L.Steiner, G.Blum, Y,Friedmann and A.Levitzki, Eur.J.Pharmacol, in press.*
2. *Cell permeable conjugates of peptides for inhibition of protein kinases. N.Livnah, A.Levitzki et al. WO 2004/110337A2*
3. *A novel substrate mimetic inhibitor of PKB/Akt inhibits prostate cancer tumor growth in mice by blocking the PKB pathway. P.Litman et al. , submitted.*
4. *EGF receptor-targeted synthetic double-stranded RNA eliminates glioblastoma, breast cancer and adenocarcinoma tumors in mice. A.Shir and A.Levitzki, PloS Med. 2006 Jan 3 (1):Epub 2005 Dec 6.*
5. *The eradication of EGFR over-expressing metastatic tumors by the systemic application EGFR targeted Poly Inosine:Cytosine. A.Shir and A.Levitzki, in preparation.*

Kinases and proteases in cancer cell invasion

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There are many unanswered questions about the metastatic process. How and when do tumor cells acquire the ability to be motile, and to degrade ECM, and can these two properties be separated? Do “cancer stem cells” already have these properties, or are they acquired later? What exactly is the role of the MMPs? What about other classes of protease? How common is the newly described protease-independent invasion of tumor cells through amoeboid movement? Are there yet other mechanisms of invasion?

Our recent research has focused on structures on the ventral surface of cells called podosomes (also called invadopodia). Podosomes are found in some normal cell types, such as osteoclasts, macrophages and endothelial cells, as well as in some cancer cells. These cells all have an intrinsic ability to degrade ECM and cross tissue boundaries. Both tyrosine and serine kinases have been implicated in regulating podosome formation, and several types of protease are concentrated in these structures. We have recently identified a scaffolding or adaptor protein, called Tks5, which is required both for the formation of podosomes, and for invasion in cancer cells. I will discuss podosome-mediated invasion, the potential use of podosome proteins such as Tks5 as markers of invasive disease, and whether these structures might contain validated targets for therapeutic intervention.

Modeling prostate cancer progression and therapy by tissue regeneration

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Epithelial cancers of the prostate can progress through a series of genetic changes eventually leading to aggressive androgen independent growth with lymph node and bone metastasis. We have established a murine prostate regeneration system to define the cellular elements critical for organ growth and response to alterations in signal transduction pathways. Tissue grafts can be formed in a short period (6-8 weeks) and assessed for conversion to cancerous behavior after genetic changes are introduced by lentiviral vectors. In particular, we have concentrated on defining the interaction and synergies between activation of the PTEN-PI3K-AKT pathway, androgen receptor signaling, and cell surface tyrosine kinase receptors as representative of events for which specific targeted therapies are possible. Our results show that naïve epithelia can be driven to androgen independent prostate cancer in this in vivo tissue reconstruction model when either activated AKT or a strong receptor tyrosine kinase signal is coupled with enhanced androgen receptor signaling. This system is useful for the assessment of therapies aimed at specific kinases or steroid receptors.

Role of MAPK pathway oncoproteins in thyroid cancer pathogenesis and as drug targets

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Cancers derived from thyroid follicular cells (papillary thyroid cancer; PTC) are the most common endocrine malignancy. Exposure to ionizing radiation during childhood confers predisposition to these tumors, as highlighted by their increased incidence after the Chernobyl nuclear accident. The signature genetic defects in radiation-induced cancers are intrachromosomal inversions linking the promoter and N-terminal domain/s of unrelated gene/s to the C-terminus of the tyrosine kinase receptors *RET* or, less commonly, *NTRK1.* The resulting chimeric genes encode for several *RET* and *TRK* oncogenes, which display constitutive tyrosine kinase activity. A novel intrachromosomal inversion involving the *BRAF* gene (*AKAP9-BRAF*) was recently found in PTCs from Chernobyl, resulting in the expression of a constitutively active BRAF chimeric protein that lacks the N-terminal RAS-binding domain. Thus, PTC either have mutations in the TK receptors *RET* and *NTRK,* or in *RAS* or *BRAF,* with no overlap between them, and these altogether account for about 70% of tumors, with *BRAF* mutations being the most common in patients with no history of exposure to radiation. In radiation-exposed patients these genes are mostly activated by genomic rearrangements, whereas in sporadic cases point mutations predominate. The fact that these oncogenes are epistatic in thyroid cancer provides compelling genetic evidence that thyroid cell transformation to PTC takes place through constitutive activation of effectors along the RET-RAS-BRAF signaling pathway. Targeted expression of each of these oncoproteins in thyroid cells of transgenic mice gives rise to murine PTCs that resemble closely their human counterparts. The significance of the RET-RAS-BRAF pathway in thyroid cell transformation is supported by functional experiments: hence, RET/PTC requires RET-Y1062 and association with Shc for transformation in vitro, and transforming effects are also disabled by knockdown of BRAF or by MEK inhibitors. Based on these observations, distal inhibitors of the MAPK pathway would be logical candidate therapeutic agents for most patients with PTC. Preclinical studies support this, as the RAF inhibitors serafinib, AAL881 and LBT613 are potent inhibitors of growth of human PTC cell lines harboring either *RET* or *BRAF* mutations. However, a phase 2 clinical trial for serafinib in PTC was completed recently, and the beneficial effects are at best modest. The same drug was also disappointing in clinical trials for melanomas, despite the fact that most melanomas also harbor

non-overlapping mutations of *RAS* or *BRAF*. The explanation for this is unknown, but includes the possibility that the compound may not be adequately concentrated in tumor tissues. Alternatively, feedback mechanisms may have dampened RAF inhibition of MAPK, or other signaling pathways may drive tumor growth once RAF-MEK-ERK is blocked.

Medullary thyroid cancers arising from thyroid parafollicular C cells are quite distinct from PTC genetically and in their biological behavior. Germline activating point mutations of *RET* confer predisposition to different familial syndromes of MTC. In addition, up to half of sporadic MTCs harbor somatic *RET* mutations. However, and by contrast to PTC, RET-mediated transformation of thyroid cells is not likely to require signaling via RAS-RAF-MEK, as RAS activation induces growth arrest and promotes C cell differentiation. The RET kinase has emerged as a promising target for therapy of MTC. Several RET kinase inhibitors have demonstrated effectiveness in preclinical models, and a phase 2 clinical trial with ZD6474 has shown promising early responses. MTCs secrete calcitonin (CTN), a useful indicator of tumor burden. Interestingly, antagonists of RET kinase result in effects on plasma CTN that are either disproportionate or dissociated from effects on tumor mass, because RET kinase mediates a physiological pathway controlling CTN secretion. Indeed, the role of traditional tumor biomarkers may need to be reassessed as targeted therapies designed against oncoproteins with key roles in pathogenesis are implemented.

Hairpin RNA screening for tyrosine kinase dependency in ovarian cancer cells

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Advanced ovarian cancer represents a particularly difficult therapeutic problem. While treatment with platinum/taxane-containing chemotherapy is often effective when first used, resistance is the standard outcome, and long term survival is uncommon. Therefore, a search for new target proteins around which specific targeted therapy might be designed is warranted. In this vein, using a small series of standard ovarian cancer cell lines. We have mounted a lentivirus-based shRNA screen directed at each of the 92 tyr kinases represented in the human genome. Each kinase was targeted by 3 or more non-overlapping hairpins. The results, thus far, point to a role for a small number of specific kinases in maintaining the viability (as measured in an ATP abundance assay) of multiple cell lines. One protein, erbB3, was found to be a particularly prevalent viability-maintaining element in these assays. In each of the lines in which erbB3 dependency was detected, Y1289P erbB3 –an activated form of the protein-was identified in complex with the p85 regulatory subunit of PI3 kinase, suggesting a role for the protein in activating PI-3 kinase and the PI3K pathway, which was also confirmed. The presence of p85-associated, (Y1289P) activated erbB3 in a growing series of primary, fresh, purified ovarian cancer cell collections, each obtained at paracentesis from a different Dana-Farber patient with malignant ascites has also been detected and will be discussed.

Targeting cyclin-dependent kinases: how different are we from yeast?

Mariano Barbacid

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We and others have previously reported that none of the four kinases directly implicating in driving interphase during mammalian cell division, Cdk2, Cdk3, Cdk4 and Cdk6, are essential for the cell division. *In vitro*, mouse embryonic fibroblasts (MEFs) derived from mice lacking each of these enzymes proliferate normally. *In vivo*, they are not required for embryonic development or normal homestasis. Exceptions include the essential requirement of Cdk4 for postnatal proliferation of specialized endocrine cells and for proper animal size. It should also be noted that Cdk2 is essential for meiosis but not for mitotic cell division.

It has been argued that these observations are due to functional compensation by other Cdks. Indeed, concomitant ablation Cdk4 and Cdk6 in the mouse germ line results in embryonic lethality due to limited proliferation of hematopoietic precursors, particularly those of erythroid lineage. Yet, *Cdk4*$^{-/-}$;*Cdk6*$^{-/-}$ embryos display normal organogenesis and cell proliferation in all other tissues examined. Moreover, MEFs lacking both of these kinases previously thought to be essential from cell cycle re-entry (G0/G1 transition), respond well to mitogens and engage in DNA synthesis with normal kinetics. Thus Cdk4 and Cdk4 play compensatory roles but only in hematopoietic cells. Likewise, Cdk6 and Cdk2 do not exhibit compensatory activities since double mutant mice only display those defects observed in each of the parental strains. We have obtained similar results with mice lacking Cdk4 and Cdk2. A significant number of double mutant *Cdk4*$^{-/-}$;*Cdk2*$^{-/-}$ mice successfully complete embryonic development. Although these mice die shortly after birth of cardiac failure, due to reduced number of cardiomyocytes in their hearts, all other tissues, including those of hematopoietic origin display normal proliferation rates. In culture, *Cdk4*$^{-/-}$;*Cdk2*$^{-/-}$ MEFs become immortal, display robust pRb phosphorylation and have normal S phase kinetics. Acute ablation of *Cdk2* in *Cdk4*$^{-/-}$ MEFs has little effect on cell proliferation. More importantly, conditional ablation of *Cdk2* in adult mice lacking *Cdk4* does not result in obvious abnormalities or decreased proliferation rates in any of the tissues analysed. Moreover, the livers of these double mutant mice regenerate normally after partial hepatectomy.

At this meeting I will also report the generation of embryos lacking all interphase Cdks. *Cdk4*$^{-/-}$;*Cdk6*$^{-/-}$;*Cdk2*$^{-/-}$ (*Cdk3* is already mutated in these inbred strains) embryos develop well until midgestation and display normal cell proliferation and organogenesis. However, they do not survive further due to lack of proliferation of hematopoietic cells due to the absence of Cdk4 and Cdk6. This defect appears to be somewhat exacerbated by the absence of Cdk2. *Cdk4*$^{-/-}$;*Cdk6*$^{-/-}$;*Cdk2*$^{-/-}$ MEFs proliferate in culture and become immortal upon serial passage. However, these cells display an extended interphase and proliferate more slowly than wild type MEFs or MEFs expressing just one interphase Cdks. Although pRb is phosphorylated at well known sites in these triple knock out cells, it is not properly inactivated since infection with the T121 fragment of SV40 T antigen restores normal proliferation rates. In summary, genetic interrogation of the role of Cdks in the mammalian cell cycle has revealed that these cells can proliferate, at least during embryonic development with just one Cdk, Cdk1. Moreover, these observations have indicated that the other Cdks do not play essential or even major compensatory roles during interphase, except in specific cell types. Thus, the generation of additional Cdks during evolution from unicellular to complex eukaryots was due to ensure adequate levels of cell proliferation in specific cell types but not to specifically drive the various phases of the cell cycle as previously proposed.

Interference with developmental and metabolic pathways for glioblastoma treatment

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In a genetic study comparing deletion pattern on chromosome 1p between glioblastoma and oligodendroglioma, we detected a unique and highly consistent recombination site on 1p11 that involved the developmental gene Notch2. By studying the role of Notch2 in brain tumorigenesis, we found that gene expression of tenascin-C is regulated by Notch2. Tenascin-C is an extracellular matrix glycoprotein that is expressed in the stroma of most solid tumors, including GBM. In another study, we assessed whether the metabolic pathway represents a potential target for cancer treatment in combination with anti-cancer drugs. The glycolytic inhibitor 2-deoxyglucose (2-DG) was used to exert a metabolic stress onto the cancer cells, reducing the availability of ATP. 2-DG alone was not able to induce cell death and had only a cytostatic effect at dose ranges from 1-25 mM. In contrast, histone deacetylase inhibitors like trichostatin A or sodium butyrate were able to efficiently trigger apoptosis. 2-DG was used as a sensitizer for apoptosis following treatment with histone deacetylase inhibitors, since 2-DG strongly inhibits protein expression including p21. Synergistic induction of apoptosis was observed in glioma and other cancer cell lines upon this combined treatment.

Mutated JAK2 and myeloproliferative disorders

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An acquired somatic mutation in the *JAK2* gene resulting in a valine to phenylalanine substitution at position 617 (JAK2-V617F) has been found in the majority of patients with myeloproliferative disorders (MPD). These are clonal disorders of hematopoietic stem cells or progenitors and result in increased proliferation of the erythroid, myeloid and frequently also the megakaryocytic linage. The JAK2-V617F mutation is located in the “pseudo-kinase” domain of JAK2, which physiologically exerts an inhibitory effect on the kinase domain. The precise mechanism of how the V617F mutation leads to a de-repression of the kinase activity is currently unknown. In vitro studies show that the JAK2-V617F protein is hypersensitive to stimulatory cytokines, but not fully factor-independent and expression of the mutated JAK2 in mouse models resulted in increased white blood cell numbers and red cell mass. Therefore, JAK2-V617F represents an attractive drug target for the treatment of patients with MPD. However, a number of observations suggest that JAK2-V617F in patients with MPD may be acting in concert with mutations in as yet unknown gene(s). The current state of the knowledge will be discussed.

Linking somatic genetic alterations in cancer to therapeutics

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Somatic genetic events are linked to the initiation and progression of cancer. Over the past decade it has become clear that such somatic alterations also establish cancer dependence that can be exploited for therapeutic gain. Our group has approached the systematic identification of somatic changes in the genomes of primary tumors and cancer cell lines using high-density SNP arrays to characterize structural alterations and high-throughput exon resequencing to identify nucleotide level alterations. These efforts have led to the identification of activating mutations in EGFR in lung adenocarcinoma
.
Using high-density SNP arrays and integrated expression analysis to interrogate the NCI60 panel of cancer cell lines we have identified gene amplification of the microophthalmia transcription factor (MITF) in malignant melanoma. Such amplification is associated with poor survival in metastatic melanoma. MITF is a known master regulator of the melanocyte lineage and thus the gene amplification event appears to maintain a normal requirement of MITF for melanocyte survival. Thus, MITF amplification appears to be a manifestation of lineage addiction. Together with BRAF V600E, MITF is capable of transforming immortalized human melanocytes.

Finally, attempting to link directly genetic alterations to therapeutic dependence, we have developed in silico supervised analysis methods for interrogating the NCI60 chemosensitivity data. Together with the Rosen lab, we found that BRAF mutant cell lines were exquisitely sensitive to MEK inhibition while RAS mutant cell lines were not

Together these data suggest that systematic approaches exploiting genome-scale data sets can unveil therapeutically tractable cancer dependencies.

Screen of the full protein kinase gene family for somatic mutations

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We have sequenced the coding exons of the family of 518 protein kinases, ~1.3Mb DNA per cancer sample, in 210 cancers of diverse histological types. Despite the screen being directed toward the coding regions of a gene family that has previously been strongly implicated in oncogenesis, the results indicate that the majority of somatic mutations detected are “passengers”. There is considerable variation in the number and pattern of these mutations between individual cancers, indicating substantial diversity of processes of molecular evolution between cancers. The imprints of exogenous mutagenic exposures, mutagenic treatment regimes and DNA repair defects can all be seen in the distinctive mutational signatures of individual cancers.

This systematic mutation screen and others have previously yielded a number of cancer genes that are frequently mutated in one or more cancer types and which are now anticancer drug targets (for example BRAF, PIK3CA, and EGFR). However, detailed analyses of the data from our screen additionally suggest that there exist a large number of additional “driver” mutations which are distributed across a substantial number of genes. It therefore appears that cells may be able to utilise mutations in a large repertoire of potential cancer genes to acquire the neoplastic phenotype. However, many of these genes are employed only infrequently. These findings may have implications for future anticancer drug development.

Genomic analyses of human cancer

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It is widely accepted that human cancer is a genetic disease caused by accumulation of mutations in oncogenes and tumor suppressor genes. These tumor-specific mutations provide clues to the cellular processes underlying tumorigenesis and have proven useful for diagnostic and therapeutic purposes. To date, however, only a small fraction of the genes has been analyzed and the number and type of alterations responsible for the development of common tumor types are unknown. The determination of the human genome sequence coupled with improvements in sequencing and bioinformatic approaches have now made it possible, in principle, to examine the cancer cell genome in a comprehensive and unbiased manner. We have begun a systematic study of the cancer genome through examination of genes families involved in signal transduction. These efforts have recently identified frequent activating mutations in a number of different kinases and phosphatases not previously linked to human cancer. For example, we have found genetic alterations in the PIK3CA gene encoding the p110 alpha phosphatidylinositol 3-kinase in ~30% of colon and breast cancers, providing a rational target for therapy in a large fraction of common malignancies. The results of these studies and their extension to genome-wide analyses of human cancer will be discussed.

Blocking the IGF-IR/PI3K survival signaling pathway in cancer cells

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Insulin-like growth factor I receptor (IGF-IR) is a membrane-bound tetramer ($\alpha\beta\beta\alpha$) with intrinsic tyrosine kinase activity. The binding of IGF-1 to the extracellular domain of IGF-IR results in the autophosphorylation of each β-subunit at specific tyrosine residues within the intracellular kinase domain and outside the catalytic domain. The activation of the receptor – through docking and/or phosphorylation of several transduction molecules – triggers the activation of dowstream signaling pathway, of which the so-called anti-apoptotic phosphatidiylinositol 3-kinase / protein kinase B (PI3K/PKB) pathway appears to have a major role in the mediation of IGF-IR biological functions.
Several lines of evidence have linked IGF-IR activation and downstream signaling to human tumor biology, implying that the blockage of IGF-IR or other components of the survival pathway in tumor cells could result in therapeutic benefits for a variety of malignancies. As an initial drug discovery strategy for blocking the IGF-IR/PI3K signaling pathway, we targeted the IGF-IR intracellular kinase domain by interfering with ATP binding. Due to the high level of sequence conservation and structural homology between IGF-IR and the insulin receptor, a major medicinal chemistry effort was devoted to achieve selectivity against the insulin receptor. Our lead discovery and optimisation strategy resulted in the identification of pyrrolo[2,3-*d*]pyrimidine derivatives that potently inhibit the kinase activity IGF-IR, and distinguish at the cellular level IGF-IR from the insulin receptor and other tyrosine kinases. Selected compounds inhibit IGF-I mediated survival and anchorage-independent growth of tumour cells in cellular settings, and impair IGF-I-induced responses and tumour growth *in vivo* after oral administration. Further efforts in our department have been devoted to inhibition of key components of this pathway – PI3K and 3-phosphoinositide-dependent protein kinase-1 (PDK1) – to tackle other genetic alterations frequently observed in solid tumors. This presentation will detail the design efforts of this program and will highlight the evolution of our IGF-IR/PI3K-targeted therapeutic strategies.

Targeting PI3 Kinases in Cancer

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Phosphatidylinositol 3-kinase (PI3K), once an “artifact” in oncogene immunoprecipitates, is now a prime target for therapeutic intervention in a number of human tumor types. PI3Ks form a complex family consisting of three classes, each with multiple subunits and/or isoforms. Only class IA PI3Ks have been clearly implicated in human cancer. Class 1A PI3Ks are a collection of p85/p110 heterodimeric lipid kinases that generate lipid second messenger in response to growth factor stimulation and subsequent activation of receptor tyrosine kinases (RTKs). The p110 catalytic subunit consists of three isoforms (α, β and δ) that are encoded by the genes *PIK3CA*, *PIK3CB* and *PIK3CD*, respectively. While p110δ is primarily found in leukocytes, p110α and p110β are ubiquitously expressed. However, we have very limited understanding of the extent to which these p110 isoforms have overlapping or distinct functions. Recently, frequent somatic activating mutations have been identified in human cancers in p110α, but not in p110β or p110δ. While only one isoform of PI3K, PIK3CA, is frequently mutated in human tumors, this family of enzymes is believed to serve as points of convergence for oncogenic signals resulting from the loss of PTEN or the activation of one of several RTKS such as Her2. Recently there has been a breakthrough in the design of PI3 kinase inhibitors; several companies and academic labs have succeeded in creating inhibitors specific for particular Class IA and other PI3K isoforms. Since isoform-specific PI3K inhibitors may display less toxicity than pan-inhibitors, the feasibility of developing such isoform specific inhibitors makes it extremely important to determine which isoforms are actually required for tumor growth.

To address the issues of isoform specificity, the Zhao lab and Roberts lab have constructed a series of reagents designed to probe the role of each isoform in both physiological growth and in pathological states. To the end, we have recently generated conditional knockout animals for the PIK3CA and PIK3CB genes via the Cre/loxP recombination system. To study gain of function in these enzymes, we have created a synthetic orthotopic human tumor system optimized for the study of kinases and used it to study PI3K isoforms. In addition, we have generated transgenic mice in which tumor formation is driven by PI3Ks. Finally, the Zhao lab has constructed kinome wide libraries of all activated serine/threonine kinases and used them to further dissect signaling downstream from PI3Ks. The first results from each of these systems will be described.

Structural Studies of Protein Kinase B and B-RAF

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Protein kinase B/Akt plays crucial roles to promote cell survival and mediate insulin responses. The enzyme is stimulated by phosphorylation at two regulatory sites; Thr 309 of the activation segment and Ser 474 of the hydrophobic motif, a conserved feature of many AGC-kinases (PKBβ/Akt2 residue numbering). To investigate the mechanisms of activation of PKB by dual site phosphorylation, and to understand its specificity for substrate peptides with the RxRxx(S/T)Hyd consensus motif, we have determined the structures of unphosphorylated (inactive) and phosphorylated (activated states) of the kinase domain. The activated state of the kinase was generated by phosphorylating Thr 309 using PDK1 and mimicking Ser 474 phosphorylation either by substituting an Asp for Ser 474, or by replacing the HM of PKB with that of PIFtide, a potent mimic of HM phosphorylation. Comparison with the inactive PKB structure indicates that the role of Ser 474 phosphorylation is to promote the engagement of the HM with the N-lobe, promoting a disorder-to-order transition of the αC-helix. The αC-helix, by interacting with phosphoThr 309, restructures and orders the activation segment, generating an active kinase conformation. Analysis of the interactions between PKB and the GSK3β-peptide explains how PKB selects for protein substrates distinct from those of PKA. These studies provide a framework for understanding the regulation of other AGC-kinases and for the development of specific PKB inhibitors (Yang *et al*., 2002a; 2002b). Results of recent drug discovery efforts will be discussed.

Over 30 mutations of the B-RAF gene associated with human cancers have been identified, the majority of which cluster to the glycine rich P-loop and activation segment of the kinase domain (Davies *et al.,* 2002). We have characterised the biochemical and physiological properties of 22 B-RAF mutants and integrated these studies with crystallographic investigations of the kinase domain of wild type and oncogenic mutant forms of B-RAF in complex with the RAF inhibitor BAY43-9006. Eighteen of 22 B-RAF mutants analysed have elevated kinase activity and signal to ERK in vivo. Surprisingly, three mutants have reduced kinase activity towards MEK in vitro, but activate C-RAF *in vivo* enabling them to signal to ERK in cells. The crystal structure of the kinase domain of both wild type and the oncogenic mutant ^{V599E}B-RAF in

complex with BAY43-9006 shows that the activation segment is held in an inactive conformation by association with the P-loop. The clustering of mutations to this region suggests that they disrupt this interaction converting B-RAF into an active conformation. The high activity mutants are able to signal to ERK by directly phosphorylating MEK, whereas the impaired activity mutants mediate MEK activation by activating endogenous C-RAF, possibly through an allosteric or transphosphorylation mechanism (Wan *et al*., 2004).

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Session X - 1

TOR signaling and control of cell growth

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TOR (target of rapamycin) is a highly conserved serine/threonine kinase that controls cell growth and metabolism in response to nutrients, growth factors, cellular energy, and stress (1). TOR was originally discovered in yeast, but has since been shown to be conserved in all eukaryotes including yeast, plants, worms, flies, and mammals. The discovery of TOR led to a fundamental change in how one thinks of cell growth. It is not a spontaneous process that simply happens when building blocks (nutrients) are available, but rather is a highly regulated, plastic process that is controlled by TOR-dependent signaling pathways. TOR is found in two structurally and functionally distinct multiprotein complexes, TORC1 and TORC2. The two TOR complexes, like TOR itself, are highly conserved. Yeast TORC1 is rapamycin sensitive, and contains KOG1, LST8 and either TOR1 or TOR2. The mammalian counterpart of TORC1, mTORC1, contains raptor (mKOG1), mLST8, and mTOR. TORC1 in yeast and mammals mediates temporal control of cell growth by regulating several cellular processes including translation, transcription, ribosome biogenesis, nutrient transport and autophagy. Yeast TORC2 is rapamycin insensitive, and contains AVO1, AVO2, AVO3, BIT61, LST8, and TOR2. mTORC2 is also rapamycin insensitive and contains SIN1 (mAVO1), rictor (mAVO3), mLST8, and mTOR. TORC2 in yeast and mammals mediates spatial control of cell growth by regulating the actin cytoskeleton. Thus, the two TOR complexes constitute an ancestral signaling network conserved throughout eukaryotic evolution to control the fundamental process of cell growth. The physiological consequences of mTORC1 dysregulation suggest that inhibitors of mTOR may be useful in the treatment of cancer, cardiovascular disease, autoimmunity, and metabolic disorders. The study of TOR signaling demonstrates the value of model organisms in biomedical research. Data on the role of mTORC1 and mTORC2 in specific tissues will be presented.

(1) Wullschleger, S., R. Loewith, and M. N. Hall. 2006. TOR signaling in growth and metabolism. Cell 124, 471-484.

The role of the nutrient input in TSC1/2-Rheb mediated mTOR signaling

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Counter to prevailing views, recent studies from our laboratory showed that nutrient, such as amino acids (AAs), input into the mTOR/S6K1 signaling pathway is not mediated by either the tumor suppressor TSC1/TSC2 or its target, the protooncogene Rheb. In the absence of TSC1/TSC2 we found that S6K1 activation is elevated and refractile to mitogen stimulation, such as insulin, but can still be regulated by AAs. However, this is not the case for Rheb as siRNA knock-down of Rheb protein levels blocks both the insulin and AA input to S6K1. Nonetheless, withdrawal of AAs, which triggers S6K1 inactivation, has no effect on elevated Rheb-GTP levels, leading to the hypothesis that Rheb-GTP is necessary but not sufficient to drive S6K1 activation in the absence of AAs. These findings suggested that the AA input to S6K1 resided on a parallel pathway to that of the TSC1/2-Rheb axis. As earlier studies demonstrated that wortmannin, a class 1 PI3K inhibitor, blocks AA-induced S6K1 activation and AAs do not induce PKB activation, this suggested that a novel wortmannin sensitive signaling component was responsible for mediating the AA input to S6K1. These observations led us to class 3 PI3K, or hVps34, as the novel target by which these responses were mediated. In brief, ectopic expression of hVps34 drives S6K1 activation, but only in the presence of AAs, and this effect is blocked by siRNAs directed against hVps34. Moreover, stimulation of cells with AAs increases hVps34 activity as measured by the production of PI3P, the product of hVps34. PI3P mediates the recruitment of proteins containing FYVE or Px domains to endosomal membranes, with PI3P rich micro-domains acting as signaling platforms. Consistent with hVps34 mediating the AA input to S6K1, this response is attenuated by expression of a cDNA containing two FYVE domains, which bind to PI3P and block binding of proteins having either FYVE or PX domains, preventing S6K1 activation.

A review of the potential of mTOR inhibitors for the treatment of human cancers

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The role and importance of the PI3K/PTEN/AKT/mTOR pathway in the development and progression of cancer is of increasing interest to the oncology community. Indeed, aberration of signaling through this pathway can occur at many levels and is associated with the majority of human cancers. RAD001 (everolimus), currently in Phase 3 clinical trials in oncology, is an oral inhibitor of the mTOR (mammalian target of rapamycin) pathway. mTOR is a key protein kinase regulating cell growth, proliferation and survival; functioning as a sensor of growth factors, energy and nutrient levels. Hence, mTOR facilitates cell growth and cell cycle progression.

Consistent with the central role of mTOR in cell growth, RAD001 demonstrates a broad in vitro antiproliferative activity, being active against a range of human tumor cell types (including breast, lung and renal; IC50s: low/sub nM). In nutrient-replete culture conditions, RAD001 treatment results in a G1 accumulation in tumor lines deemed sensitive. Using isogenic tumor cell lines, generated to stably express either a wild type mTOR cDNA or a mutant that does not bind RAD001, it has been demonstrated that the antiproliferative effects of RAD001 are indeed through inhibition of mTOR function.

RAD001 also exhibits a potent antitumor activity at well tolerated doses in a range of experimental animal models of cancer derived from a number of tumor types. Strikingly, despite the observation that some tumor cell lines are indifferent to RAD001 in vitro (IC50s: μM), tumors derived from these lines display sensitivity to RAD001 in vivo. This occurs despite tumor RAD001 concentrations never exceeding the IC50 for inhibition of cell proliferation. These observations led to the characterization of an antiangiogenic activity which may account, at least in part, for the in vivo antitumor activity against cells considered RAD001-indifferent in vitro.

Although in experimental models RAD001 displays potent antitumor activity as a mono-agent, data is emerging which suggests that the true potential of mTOR inhibitors may rather be in combination with other therapeutic agents. Indeed, positive interactions have been demonstrated between RAD001 and standard chemotherapeutics, as well as anticancer agents targeting both growth-factor and estrogen-response pathways. For example, RAD001 enhances the induction of tumor cell death (apoptosis) when used in combination with DNA-damaging agents, receptor tyrosine kinase inhibitors or antagonists of estrogen signaling. These observations will be discussed, and the application of a biomarker strategy aimed at optimizing dose evaluation and patient selection in the clinic will be presented.

MAPKAP Kinase-2 is a critical regulator of cell cycle progression in tumors after DNA damage

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In response to DNA damage, eukaryotic cells activate a complex signaling network to arrest the cell cycle and initiate DNA repair. In addition to the well-established ATM/Chk2 and ATR/Chk1 pathways that contribute to this response, we have identified a third DNA damage signaling pathway mediated by p38 MAPK-activation of MAPKAP Kinase-2 that is required for tumor cell survival after DNA damage. UV-induced MAPKAP Kinase-2 activity is necessary for cells to execute both the G1/S and G2/M checkpoint. In response to cisplatin, MAPKAP Kinase-2 is required for Cdc25A destabilization and activation of the G1 and intra-S checkpoints, while in response to doxorubicin or camptothecin, MAPKAP Kinase-2 is required for targeting of Cdc25B to 14-3-3, and activation of the G2/M checkpoint. MAPKAP Kinase-2 depletion abolishes these cell cycle checkpoints in p53-defective tumor cells, sensitizes them to chemotherapy and UV irradiation in culture, and induces dramatic tumor regression after low-dose chemotherapy in a murine xenograft tumor model. Chk1 responds to the same genotoxic stresses that activate MAPKAP Kinase-2, and Chk1 is activated normally in the MAPKAP Kinase-2-depleted cells that display aberrant checkpoint function. Thus, it appears that MAPKAP Kinase-2 and Chk1 function together as a molecular ‘AND’ gate that is required to integrate DNA damage signals to control cell cycle progression and prevent mitotic catastrophe within tumors. Our data indicate that small molecule inhibitors of MAPKAP Kinase-2 will function as potent tumor-specific chemosensitizing agents and anti-cancer drugs.

Mec1 kinase recruits DNA damage to the nuclear pore

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Evidence from both yeast and mammalian cells supports the notion that double strand breaks aggregate at sites of repair in vivo, and predicts that chromatin should be able to move throughout the nucleus to promote this interaction. Genetic analyses have shown that interchromosomal translocations between homologous sequences are stimulated if both sequences experience a DSB, consistent with the notion that the break sites are brought together in repair center. In budding yeast, if repair by recombination is not immediate, the ATR/ATRIP homologues Mec1/Ddc2 are recruited to the DSB and coat ssDNA for up to 5 kb. The focus then moves to the nuclear periphery, where it associates with nuclear pore complexes and not with telomeres. This relocalization of the DSB is dependent on Mec1 kinase, and the Mec1-medicated pore association of the DSB is compromised in a *slx8* mutant. A proteomic/synthetic lethal approach suggested that a complex containing putative de-SUMOlation proteins Slx8 and Slx5 is associated with the nuclear pore. Slx8 and Slx5 form a complex with nuclear pore components Nup133, Nup84 and Nup60, and mutations in any of these components increase spontaneous gross chromosomal rearrangements. Finally, spontaneeous recombination rates can be increased by artifically tethering sites to the nuclear periphery. We will examine the role of Mec1 phosphoinositol binding in both the relocalization and in repair events. We propose that DSB targeting to the nuclear periphery in yeast is an active process signalled, if not directly mediated, by the Mec1 kinase.

PKC epsilon controls the final committed step in cell division

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The cell cycle is an exquisitely controlled process, engaging many regulatory inputs to ensure precision in its completion. The final step in this process of cell duplication, the ultimate separation of daughter cells, is controlled by a member of the PKC gene superfamily. It has been established that PKCε displays a specific pattern of phosphorylation at mitosis that reflects a requirement for its activation through 14.3.3 complex formation. The multiple regulatory pathways engaged in the execution of these newly identified PKCε phosphorylation sites have been defined, as has the functional activation associated with 14.3.3 binding. The expression of phosphorylation defective, or 14.3.3 binding defective, PKCε demonstrates that efficient cell separation requires assembly of this activated PKCε complex. Specific inhibition of PKCε activity using a chemical genetic approach independently demonstrates this PKCε requirement. It is concluded that this specific PKCε complex controls this final separation step in cytokinesis.

NVP-AEB071: first protein kinase C inhibitor prolonging graft survival

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Inhibition of T-cell activation is the most efficient way to prevent transplant rejection. Among current immunosuppressants, only calcineurin inhibitors (CNI) inhibit T-cell activation, but their long term use is associated with side-effects. AEB071 is a novel selective PKC inhibitor preventing T-cell activation *via* a calcineurin-independent pathway. Here, we present a summary of preclinical data including *in vivo* models in rodents and non-human primates as well as Phase I clinical results obtained with AEB071.

Structural biology contributions to tyrosine kinase drug discovery projects

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The structures of protein kinases are highly conserved, since the positions of residues important for catalytic activity are constrained by the requirements of the phospho-transfer reaction. Therefore, homology modelling frequently serves as a good basis for the design of competitive inhibitors binding to the ATP site. However, the structures of inactive kinases have surprisingly different conformational states, which are related to the variety of different mechanisms of regulation and the inherent flexibility of this family of proteins, and these are much less easy to predict. When designing inhibitors, selectivity can be gained by targeting a specific inactive conformation of the kinase, as Glivec® has shown. In this presentation the methods of regulation and the resulting conformational states will be presented for a range of tyrosine kinases. It will also be shown how this structural information had an influence on assay design and how it can be used in drug discovery.

Protein kinase inhibitors by design

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Inhibiting the catalytic activity of protein kinases has become one of the major therapeutic concepts in contemporary drug discovery. The first protein kinase inhibitors were identified by screening more than a decade ago. From that time, the intense activity of structural biologists in the field, has given us access to hundreds of crystal structures of protein kinases (apoenzymes or ligand complexes). Concomitantly, a lot of experience has been gained in the structure-activity relationships of protein kinase inhibitors. The combined information has provided us with a deep insight into the structural determinants of kinase inhibition by small molecules binding to the ATP (cofactor) pocket. We will present and illustrate how this knowledge can be exploited to design by molecular modeling new kinase inhibitors.

Multi-targeted kinase inhibitors: an old (or new) paradigm?

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Protein kinases play an essential role in many signaling pathways and have the potential to contribute to diseases ranging from cancer and inflammation to diabetes, cardiovascular as well as other disorders which makes this protein family the most populated class of drugable targets in the pharmaceutical industry.

The finding of an association of a set of gain of function (GOF) mutations in the protein tyrosine kinases like Kit, PDGFR, Fms, Bcr-Abl, the FGF-Rs, Flt-3 and Alk in various malignant disorders make these kinases ideal targets for validating the clinical utility of protein kinase inhibitors as therapeutic targets. In addition, these GOF mutations have allowed to better understand the mode of binding of kinase inhibitors as well as how a kinase can be resistant to the action of the kinase inhibitor.

In this lecture case studies of drug development will be discussed – with special emphasis on the so called "multitargeted kinase inhibitors" - which will provide additional insights regarding strategies that are being employed to generate second generation kinase inhibitors.

GOF mutation of Kit and PDGFR are in part responsible, amongst other, for a subset of gastrointestinal tumors (GIST) which are sensitive to Imatinib treatment and which become eventually resistant to Imatinib while a particular GOF mutation in Kit which is associated with aggressive mastocytosis is resistant to Imatinib. All of these GOF mutations of Kit and PDGFR are sensitive to PKC412 (midostaurin). This multitargeted kinase inhibitor has also shown to be effective in malignant diseases driven by FGF-R1 and Flt-3 both in preclinical as well as clinical settings. GOF mutations in the Flt-3 receptor are a poor prognostic factor for a subset of patients with acute myelogenous leukemia (AML). In AML, midostaurin has shown to effectively inhibit the effects mediated by the GOF mutations of Flt-3 in vitro, in vivo and in the clinic indicating the utility of this agent in the treatment of AML.

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