FUTURE ONCOLOGY

TECHNOLOGY, PRODUCTS, MARKETS AND SERVICE OPPORTUNITIES

A NEW MEDICINE PUBLICATION JUNE 15, 2005 VOLUME 8, NUMBER 5

MEETING COVERAGE

TARGETING FOR CANCER IMAGING AND THERAPY

FROM THE "IMMUNE, VASCULAR AND GENE TARGETING FOR CANCER IMAGING AND THERAPY" MEETING ORGANIZED BY THE SIDNEY KIMMEL CANCER CENTER, IN SAN DIEGO, CA. FEBRUARY 28-MARCH 2, 2005

MECHANISMS IN MALIGNANCY

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This report has been prepared from the "Immune, Vascular and Gene Targeting for Cancer Imaging and Therapy" Meeting organized by the Sidney Kimmel Cancer Center in San Diego, CA, February 28 to March 2, 2005.

DELIVERY OF DRUGS TO TUMORS

One of the most challenging aspects of today's drug development efforts is delivery of effective doses of anticancer agents selectively to tumor sites sparing healthy tissues.

Enhancing the Delivery of Drugs to Tumors

Stem cells represent a new technology for the diagnosis and treatment of cancer, and can also be used like a vector to deliver genes to tumor cells. Stem cells may penetrate into the tumor to deliver a suitable payload.

Nagy Habib, PhD, from Imperial College and Hammersmith Hospital (London, UK), discussed the potential of using adult stem cells to treat patients with cancer. Stem cells taken from a patient may be manipulated to generate various lineages of functional cells. Within 10 to 12 days after removal from the host, a 2-log increase in cell numbers may be achieved *ex vivo*; addition of growth factors allows cells to further proliferate without plateauing. *In vivo*, stem cells can penetrate into the liver of a mouse, thereby setting the stage to test their therapeutic potential for halting micrometastases in the human liver.

A phase I clinical trial, administering a growth factor prior to an injection of stem cells, was conducted to test this possibility. A phase II clinical trial is now being planned to administer ascending doses of stem cells. Once it is shown that stem cells penetrate into the tumor, the question remains of what should then be delivered, presenting researchers with an exciting range of possibilities.

Multivalent monoclonal antibodies (MAb) may be preferable to those focusing on a single target (ligand), according to King Li, PhD, of the National Institutes of Health (NIH; Bethesda, MD). It may be more appropriate to employ a methodology that mimics the process by which the host's own products enter a cell that involves many interactions with multiple cell receptors. Therefore, MAb engineering should focus on multivalent agents that interact with many receptors on the endothelium, thus increasing the percentage of the MAb that actually interact with the tumor.

Polymerized vesicles (PV) may meet the design goals of targeted drug delivery that ideally requires encapsulation of a drug in a biocompatible particle, to allow for long intravascular confinement. A PV is a hollow nanoscale sphere with a high affinity metal binding capability on the outside surface, incorporating MAb or ligand elements. In addition, a drug of choice can be inserted into the hollow sphere for targeted drug delivery. Metals chelated to PV include indium and yttrium-90, used for imaging. The unique feature regarding PV is that it represents a single particle for both imaging and treatment, with many therapeutic applications.

According to Dr. Li, multivalent PV combined with magnetic resonance (MR) imaging and gamma scintigraphy, may be used to image protein expression *in vivo,* and/or design imaging agents of vascular molecular receptors. The *in vivo* specificity of the targeted molecular imaging agents has been validated in animal models. Because PV are designed to carry either contrast or therapeutic agents or both, vascular-targeted imaging may be used in selecting patients for treatment and guiding vascular-targeted therapies. Using this combined vascular-targeted imaging and therapy approach, personalized treatment may potentially be delivered, maximizing efficacy and minimizing side effects (Li KC and Bednarski MD, J Magn Reson Imaging, Oct 2002;16(4):388-93).

Pulsed high-intensity focused ultrasound (HIFU) is a novel means of enhancing drug delivery to tumors by breaking down barriers preventing tumor infiltration. HIFU is a noninvasive approach that uses high frequency focused ultrasound energy to destroy biologic tissues such as tumors, at a depth of typically 1 to 10 cm, without affecting intervening anatomical structures. HIFU has been evaluated in the treatment of prostate, bladder, pancreatic, kidney, and liver cancer.

Within tumors, high pressure makes it hard for liquids to leak; therefore, a mechanism is desirable that would facilitate drug access to tumors. Using scientific principles relating to chemically engineered materials, Dr. Li hypothesized that physical energy could open barriers leading to tumor infiltration. Pulses of ultrasound were applied to produce kinetic energy, but not heat. Such pulses caused tissues to fatigue, just as metals fatigue, causing junctions to open. Pulsed HIFU causes tissue to vibrate 1,000,000 times a second. Applied for 5 minutes over an established squamous cell carcinoma (SCC) in a mouse, pulsed HIFU increased delivery of docetaxel by more than 100% without damaging any of the surrounding tissues.

TC1 MAb was designed to target caveolae that are plasmalemmal microdomains involved in vesicular trafficking and signal transduction. Jan Schnitzer, PhD, of the Sidney Kimmel Cancer Center, focused on the therapeutic potential

of targeting caveolae. Although some success has been reported in the case of soft tumors, imaging and treatment of solid tumors has been hindered despite the fact that small molecules have almost universal cellular access. Delivery and tumor targeting with MAb is even more challenging. For these reasons, new targets and ways to penetrate into the tumor need to be developed. At the same time, a rational means of investigating the numerous targets already available needs to be addressed. For this reason, a hypothesis-driven biology paradigm was used to identify a small subset of proteins induced at the tissue/blood interphase that is accessible to intravenously (IV) administered MAb. Subcellular fractionation, subtractive proteomics, and bioinformatics were used to identify endothelial cell surface proteins exhibiting restricted tissue distribution and apparent tissue modulation. This analytical strategy can map tissue- and disease-specific expression of endothelial cell surface proteins to uncover novel accessible targets useful for imaging and therapy (Oh P, etal, Nature, 10 Jun 2004;429(6992):629-35; comment in: 618-9). By isolating luminal endothelial cell plasma membranes of various solid tumors, 83 unique proteins have been identified, many discovered via proteomic analysis of endothelial caveolae.

Caveolae are small plasmalemmal invaginations that actively pump MAb into the tumor. Targeting caveolae, therefore, represents a novel means of helping therapeutics penetrate into the tumor. Using a multidimensional protein identification technology (MudPIT), 450 proteins with 3 or more spectra were identified in luminal endothelial cell plasma membranes isolated from rat lungs, and from cultured rat lung microvascular endothelial cells. Interestingly, 41% of proteins expressed *in vivo* were not detected *in vitro* (Durr E, etal, Nat Biotechnol, Aug 2004;22(8):985-92).

TC1 MAb, constructed using this approach to enter into lung tissue, is able to completely penetrate into a solid tumor after only 1 hour. In mice injected with solid tumors, radioimmunotherapy (RIT) targeted to caveolae increased animal survival by more than 80%. Accessible targets to this approach are expressed on the blood vessels of human breast, kidney, liver, lung, and brain cancer. Unlike MAb that target endothelial cell surface proteins not found in caveolae, MAb specific for caveolae allow for rapid targeting, imaging, and transendothelial transport within minutes.

Adoptive cell transfer (ACT) enhances targeting of T cells, and also improves their cell-killing ability. Patrick Hwu, MD, of M. D. Anderson Cancer Center (Houston, TX), discussed approaches to enhance the targeting ability of T cells. At the NIH, the response rate of 570 patients with metastatic melanoma treated with recombinant vectors was only 2%. To help increase responses, patients may be immunized at earlier stages of disease, thymic activity may be increased, CD4+ T cells may be stimulated, or T cells may be activated via adoptive cell transfer (ACT). The latter method involves removing the T cells, activating them with IL-2, and then reinjecting them into the patient. Chemotherapy allows the infused T lymphocytes to proliferate better in the patient through a mechanism referred to as lymphoid depletion. This method of activating T cells has increased the response rate of patients with melanoma to 51%.

The ability of T cells to migrate into tumor tissue can also be enhanced by engineering T cells to recognize chemokines and integrins expressed on the tumor vasculature. Because tumors express arginine-glycine-aspartic acid (RGD) fusion receptors, T cells were transduced to recognize the RGD-PSGL1 fusion protein, which in turn allowed these cells to bind to the tumor endothelium.

Dr. Hwu is also developing T cells transduced with CXCR-2, a chemokine receptor found on tumor vasculature, as well as *in vivo* models to induce trafficking, T-cell migration, and subsequent tumor destruction. Also, preclinical evaluations have led to a new potential application for dendritic cells (DC) in the *in vivo* stimulation of adoptively transferred T cells for the immunotherapy of cancer (Lou Y, etal, Cancer Res, 15 Sep 2004;64(18):6783-90). A clinical trial proposes to use T cells and DC to treat metastatic melanoma.

Gene Transfer

Lentiviral vectors, derived from human immunodeficiency virus (HIV) from which all viral genes have been removed, have been constructed for gene transfer. Farzin Farzaneh of Kings College (London, UK) discussed the use of these vectors in the treatment of acute myeloid leukemia (AML). Tumor rejection can be accomplished by activating cytotoxic T cells (CTL), expanding T cells, and blocking tumor-induced immune suppression. In particular, expression of B7.1 (CD80) and IL-2 results in AML rejection via natural killer (NK) and T-cell responses against malignant cells. The combined expression of costimulatory factors and pro-inflammatory cytokines stimulate effective immune-mediated cancer rejection in murine models. Specifically, syngeneic cancer cells genetically modified to express B7.1 induce rejection of previously established murine solid tumors; transduction with IL-2 further increases survival.

To overcome shortcomings associated with poor rates of gene transfer and inefficient expression of multiple transgenes encoded by single vectors, a third generation self-inactivating lentiviral vector has been constructed for gene transfer of B7.1 and IL-2 into AML cells. In this construct, B7.1 and IL-2 are expressed as a single fusion protein that is post synthetically cleaved by the enzyme furin to generate biologically active membrane-anchored B7.1 and secreted IL-2. This allows efficient transduction of both established and primary AML blasts, resulting in expression of the transgenes in up to 98% of the cells following a single round of infection. In whole cells taken from patients with AML, the combined expression of B7.1 and Il-2 results in allogeneic and autologous T-cell stimulation.

Stimulated lymphocytes secrete greater levels of Th1 cytokines, and show evidence of specificity, as indicated by their increased proliferation in the presence of autologous AML compared to remission bone marrow cells (Chan L, etal, Mol Ther, Jan 2005;11(1):120-31).

A pilot clinical trial (GTAC 098) was approved in November 2004, by the UK Gene Therapy Advisory Committee (GTAC), to administer lentiviral-mediated *ex vivo* transduced AML blasts expressing B7.1 and IL-2 to patients with poor prognosis AML showing signs of relapse following chemotherapy-induced remission and bone marrow transplantation (BMT). The main objective of this trial is to stimulate a more effective graft versus leukemia (GvL) effect. According to the protocol, patients' peripheral blood cells, which contain leukemic cells, are removed prior to post-remission chemotherapy and BMT, and infected *ex vivo* with the vaccine. Modified cells are lethally irradiated before being returned to patients with signs of relapse after treatment with chemotherapy and BMT. Infecting patients' leukemic cells with CD80/IL-2 renders them more recognizable to grafted, healthy, immune cells from the donor, which can then mount an effective immune response against the leukemia. The trial's objective is to determine whether lethally irradiated modified leukemic cells can stimulate a GvL effect, prolong the duration of remission, and provide a meaningful survival benefit. Currently, the prognosis for patients with AML who are 55 and older is very poor.

THERAPEUTIC APPROACHES IN DEVELOPMENT

Apoptosis Enhancing Approaches

Bcl-2 inhibits the ability of mitochondria to release pro-apoptotic proteins. Cancer cells are able to shut down the protease system that allows for cascade-induced apoptosis. Therapeutics being developed against the protein, gene, and mRNA forms of Bcl-2 include small molecule antagonists in preclinical testing, commercialized transcriptional regulators, and antisense oligonucleotides in clinical trials, respectively.

John C. Reed, MD, PhD, of the Burnham Institute (La Jolla, CA), discussed apoptosis-based therapies for the treatment of cancer. Dr. Reed described how the protein TR3 induces a unique conformational and mechanistic change in Bcl-2.

In one instance, retinoids have been shown to prevent Bcl-2 activation. Retinoids are derivatives of vitamin A that are effective in preventing lung cancer in animals, but they lack efficacy in clinical trials in cigarette smokers because nicotine induces production of TR3. The synthetic retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN) and its analogs (3-Cl-AHPC) cause apoptosis in lung cancer cells, while paradoxically increasing TR3 levels in these cells. In response to AHPN, TR3 migrates from the cell nucleus and binds to Bcl-2, a process that allows TR3 to reside on mitochondria causing cell death. In essence, TR3 converts Bcl-2 from an inhibitor to a promoter of apoptosis. These observations reveal exciting opportunities to develop novel therapies. As for prognostic significance of these markers, patients with prostate cancer who express both TR3 and Bcl-2 have a 42% survival rate at 3 years.

Genesense, an antisense oligonucleotide against Bcl-2 under development by Genta (Berkley Heights, NJ), is currently in phase III clinical trials in chronic lymphocytic leukemia (CLL), multiple myeloma, and melanoma. While results have been discouraging with Genesense in multiple myeloma and inconsistent in melanoma, promising results have been obtained in CLL.

When Abbott Laboratories (Abbott Park, IL) deduced the structure of Bcl-2, a hydrophobic pocket was found to be the site of interaction. Gossypol, a Chinese herbal medicine that induces apoptosis, interacts with this hydrophobic pocket on Bcl-2. Semisynthetic derivatives of gossypol are now being engineered with the aim of eliminating the compound's two reactive aldehyde groups that cause toxicity. By a process know as NMR-lead optimization, scientists can covalently link molecules that inhibit adjacent areas of a desired target, thereby allowing a drug to attach better. An important issue is to choose which of the 6 members of the Bcl-2 family to inhibit to obtain a maximum drug effect.

Other methods in apoptosis drug development include programs to stimulate the TNF family of death receptors 4 and 5, identify small molecule antagonists to inhibitor of apoptosis proteins (IAP), and downregulate FLIP.

Oncogene Inactivation

PSF protein and mouse VL30 (mVL30) retrotransposon RNA complexes promote transcription of previously regulated oncogenes, turning an otherwise normal cell into a malignant cell. According to Alan Garen, MD, of Yale University (New Haven, CT), this is an important effect because mVL30 RNA, which is present in virtually all mouse cells, is also present in retroviral vectors for gene transfer and gene therapy that are produced in packaging cells derived from mouse cells. The mouse genome contains multiple copies of transcriptionally active mVL30 DNA, and virtually all mouse cells contain mVL30 RNA although the level varies among different tissues, and at different developmental stages. A remarkable property of retroviral vectors is the capacity to transmit mVL30 RNA from a packaging cell to cells infected by the retrovirus, which synthesize, integrate, and transcribe mVL30 cDNA. Retroviral-mediated transfection of tissue factor (TF) cDNA into a nonmetastatic human melanoma cell line increased the metastatic potential of the cells to which mVL30-1 RNA also had been transmitted (Song X, etal, PNAS USA, 13 Jan 2004;101(2):621-6).

PSF protein, a tumor suppressor, has both RNA and DNA binding domains. These domains allow it to splice RNA like a spliceosome, and regulate gene expression by repressing transcription. In humans, when PSF combines

with the noncoding RNA regions of mVL30, transcription of previously regulated oncogenes is allowed to proceed, and an otherwise normal cell becomes malignant. In its otherwise normal state, PSF binds DNA to prevent the development of cancer. This reversible process is further supported by the discovery of PSF mutations in cancer cells. Therefore, retrotransposons such as mVL30 should not simply be thought of as 'junk' DNA, because they have acquired physiologic and pathologic functions.

Oligonucleotides against jun kinase (JNK) are being investigated in the treatment of prostate cancer. Dan Mercola, MD, PhD, of the Sidney Kimmel Cancer Center, presented preclinical data regarding antisense oligonucleotides that target JNK to treat prostate cancer. JNK activity, which controls activation of activating protein 1 (AP-1), is directly proportional to human prostate cancer cell growth. An antisense oligonucleotide to JNK 2, provided through a collaboration with Isis Pharmaceuticals (Carlsbad, CA), inhibits serum-induced growth of prostate carcinoma *in vitro*. Moreover, this antisense oligonucleotide is able to inhibit growth of prostate cells by 88% *in vivo*; better than JNK 1 antisense treatment. Combination of antisense JNK 1 and JNK 2 produced ulceration in 100% of mice. Further research will evaluate the genes controlled by AP-1 complexes.

MDX-010, under development by Medarex (Princeton, NJ), is being evaluated in a variety of solid tumors. Jeffery Weber, MD, PhD, of the University of Southern California (Los Angeles, CA), addressed current clinical research pertaining to Medarex's anti-CTLA-4 MAb MDX-010. Currently, there are two phase I clinical trials administering MDX-010 in combination with a melanoma peptide vaccine, to patients with Stage III/IV melanoma. In the first trial, 10M-00-4, MDX-010 is administered 8 times over the period of 1 year, while in the follow on trial, 10M-00-6, the drug is administered 12 times over the same period. In terms of toxicity, all Grade 3 adverse events have occurred in the gastrointestinal (GI) tract. Among 17 evaluable patients, 8 responsed, indicating an increase in CD4+.

Research revealed 4 CTLA-4 single nucleotide polymorphisms (SNP) associated with autoimmune disease onset, toxicity, and relapse. In this patient population, those who experienced little toxicity had a high chance of relapse, while the relapse rate among those patients with notable toxicity was low. In particular, 3/8 patients with autoimmune toxicity have relapsed, while 8/11 with no toxicity relapsed. Adverse events are, therefore, associated with response to treatment. Dose-limiting toxicity (DLT) was established at 3 mg/kg, and maximum tolerated dose (MTD) at 1 mg/kg, every 6 to 8 weeks. A new phase II clinical trial, MDX010-08, will administer the MAb in combination with dacarbazine (DITC) to patients with Stage IV melanoma. Though responses have been few, they persist for a significant time in these hard-to-treat patients.

Angiogenesis Inhibitors/Antimetastatic Agents

Avastin, an antagonist of vascular endothelial growth factor (VEGF), has been commercialized by Genentech (South San Francisco, CA). According to Napoleone Ferrara, PhD, of Genentech, the different isoforms of VEGF exert unique activity and binding to the 3 types of VEGF-receptors, numbered 1, 2, and 3. VEGF is essential for embryonic growth, but has limited value in adult organ and endothelial cell growth. Hypoxia, as well as various cytokines, stimulate VEGF expression. In 1993, the MAb A4.6.1, now Avastin, was shown to inhibit VEGF-mediated angiogenesis and suppress tumor growth *in vivo*. According to results from a phase III clinical trial (protocol ID: AVF2107g), the addition of Avastin to the combination of IV 5-FU/folinic acid and irinotecan (IFL regimen) resulted in a 5-month survival advantage in patients with metastatic colorectal cancer. Other phase III clinical trials are being performed in patients with lung, kidney, breast, and pancreatic cancer.

SKI-606, an inhibitor of Src, may discourage vascular permeability that appears to promote cancer metastasis (see accompanying article). David Cheresh, PhD, of the Moores Cancer Center at the University of California San Diego, discussed vascular pathways in tumors and approaches to target blood vessels. VEGF induces angiogenesis by stimulating endothelial cells in response to hypoxia, a condition that occurs in both cancer cells and ischemic injury. VEGF also increases vascular permeability, causing blood vessels to 'leak'. Dr. Cheresh's initial work did not involve the field of oncology, but rather cardiology. Ischemic injury cells turn hypoxic, leading to VEGF expression that causes vascular leak and ultimately, edema. When the process from VEGF expression to vascular leak was further examined, it was hypothesized that the Src pathway played some type of role, i.e., that VEGF stimulated Src. Research revealed that Src blockade prevented edema during ischemic disease, proof that the pathway did play an integral role in vascular permeability. Moreover, two mutations of VE-cadherin resulted in increased cell permeability, because p120 and β-catenin were able to bind at these two sites. This observation led to the discovery of an additional role played by Src. When VEGF binds to the cell receptor, Src is induced, causing the dissociation of p120 and β-catenin. Therefore, an Src inhibitor could prevent such cellular interactions, and in turn, discourage vessel permeability.

The focus of this research then turned to oncology. Because tumors produce VEGF, researchers sought to ascertain if vascular permeability has any defined role in cancer metastasis. Mice deficient in Src are protected from pulmonary metastasis. In preclinical tests, Src knockout mice follow the same course as those treated with a VEGF inhibitor. To build upon this preclinical data, mice were treated with the Src inhibitor SKI-606. The drug did not reduce tumor size, but did hinder tumor cell metastasis as long as treatment was delivered promptly after

the establishment of the tumor. Because cells expressing VEGF metastasize more efficiently than those that do not, inhibitors of VEGF that reduce vascular permeability may represent a promising option for the treatment of cancer.

Oncolytic Measles Virus

Measles virus represents a novel approach to targeted cancer therapy. Stephen Russell, MD, PhD, of the Mayo Clinic (Rochester, MN) presented information regarding the engineering of such a virus. According to Russell, no other groups are currently working on harnessing the destructive power of the measles virus to treat cancer. Issues concerning this effort include both safety and efficacy. Many have thought viruses to be too dangerous to work with, citing the possibility of new pathogens evolving from research on viruses. Moreover, in terms of clinical benefit, the immune system is known to efficiently detect and eliminate viruses. Dr. Russell chose to work with the measles virus, a negative strand RNA virus, because of its favorable safety and enticing efficacy record. The attenuated form of the measles virus is safe because nearly every person in the developed world has been vaccinated against the virus. Moreover, because the life cycle of the virus is entirely cytoplasmic and entirely RNA-based, there is no risk of chromosomal integration. In case reports, the virus displayed activity in combating cancer in patients with lymphoma who developed measles. Taken together, this historical evidence supports the development of a measles virus for cancer therapy.

Dr. Russell is developing the Edmonston measles strain (MV-Edm), originally isolated in 1954 from the throat washings of David Edmonston, a measles patient. The virus has been attenuated by being grown in cells that it would not normally infect. MV-Edm displays oncolytic activity in animal models against lymphoma, multiple myeloma, glioma, and ovarian and pancreatic cancer. The measles virus mechanism of action is fusogenic, implying that the virus is able to fuse cells together; clumping malignant cells are more prone to apoptosis. More specifically, the F and H proteins of the measles virus are responsible for fusing cells together. The H glycoprotein binds to CD46, a regulator of complement activation, which is overexpressed on human cancer cells, while the F protein triggers the actual fusion action. Experiments have revealed that the measles virus can infect cancer cells that have a low CD46 concentration, but no fusion occurs. When a high CD46 concentration is present, the virus can both infect and fuse.

Dr. Russell noted 5 distinct problems with MV-Edm, and provided a subsequent solution. The first issue regards the inability to monitor the spread of the virus. To solve this problem, Dr. Russell incorporated the carcinoembryonic antigen (CEA) gene into the measles virus (MV-CEA). This allows for detection of the virus, because a clinical protocol has already administered MV-CEA to patients with ovarian cancer. The first patient was treated in July 2004, with 5 additional patients treated to date. In this dose-escalation, phase I clinical trial, being conducted at the Mayo Clinic (protocol ID: MC0117), MV-CEA is being administered intraperitoneally (IP) to patients with recurrent ovarian cancer. The trial's objectives are to establish MDT and safety of this agent administered IP, and to assess its effects in the body using blood, urine, and throat gargle specimen(s), and tissue samples.

The second problem with the measles virus is that it is not active in all tumor models. To remedy this, Dr. Russell inserted the sodium iodide symporter (NIS) gene into the virus (MV-NIS). The NIS protein originating in the thyroid gland is responsible for uptake of iodine, which is required for normal function of the thyroid. Moreover, that same protein attracts radiolabeled iodine when it is being used to treat thyroid cancer. In an *in vivo* model of multiple myeloma, MV-NIS plus radioiodine caused tumors to regress completely. Russell has deemed such treatment 'radioviral therapy', and a phase I clinical trial is planned to administer MV-NIS IV to patients with multiple myeloma. The protocol calls for MV-NIS followed by cyclophosphamide and serial iodine 123 (^{123}I) imaging to monitor the viral spread. As may be inferred from this discussion, the NIS insert has also helped to solve the issue of detecting where the virus goes within the body.

Thirdly, the virus is not totally specific to tumor cells because CD46 is also expressed on normal cells, which may lead to toxic effects. Dr. Russell has approached this targeting issue by engineering the H protein to ablate CD46 and signaling lymphocyte activation molecule (SLAM) binding. The retargeted measles virus can now recognize CD38 or the epidermal growth factor receptor (EGFr). *In vivo*, the modified virus was able to target SCOV tumors that express EGFr, and Raji tumors that express CD38. Dr. Russell believes that vascular targets are most promising for the modified measles virus. Furthermore, he has developed a measles virus that targets APP (833-retargeted MV), displaying activity in rats with lung cancer.

The fourth point is that host-generated anti measles antibodies may impede delivery; thus, the question arises of how to circumvent these antibodies. A possible solution may be to infect cells with the modified measles virus *ex vivo*, thereby inducing the cells to deliver the virus when reinfused in the patient.

The last problem to be addressed is that an anti-measles immune response may impede the spread of the virus. Dr. Russell noted that cyclophosphamide suppresses the immune response to a measles virus to circumvent this problem.

TECHNOLOGY ADVANCES

Proteomics

FLEXGene (Full Length EXpression-ready) repository allows researchers to screen protein function for any set of genes being expressed *in vitro* or *in vivo* (Brizuela L, etal, Mol Biochem Parasitol, Dec 2001;118(2):155-65).

Joshua LaBaer, MD, PhD, of the Harvard Medical School Institute of Proteomics (Boston, MA), focused his remarks on methods to harness the human proteome. After the completion of the human genome project, the next common resource needed will be a repository representing the whole human proteome. Proteomics may be divided into two areas of focus. The first reflects the abundance of proteins in nature, and aims to identify and quantify a large number of these proteins. The second focus, which relates to Dr. LaBaer's work, is to understand and describe the function of such proteins.

The ideal comprehensive protein repository would comprise at least 1 clone per mRNA, allowing for easy genetic transfer into any vector. To construct a master clone, the coding region of interest is excised, recombination sites are added, and the construct is inserted into a plasmid vector. Using the FLEXGene repository, a master clone with the desired gene sequence, may be inserted into an expression vector when mixed with an enzyme that frees the sequence from the master clone, and the death sequence from the expression vector. This sequence then trades spots with the death sequence, and the only product left is the desired sequence in the expression clone. This recombinational approach represents a single step (1 hour), high throughput, nearly 100% efficient, universal strategy to move cDNA to any vector. Dr. LaBaer's group has been able to establish full length cDNA representing all human coding regions in a vector system that allows for transfer into any protein expression vector. Peptide tags are added to the protein so that any transfers can be visualized.

The FLEXGene repository allows researchers to screen protein function for any set of genes being expressed *in vitro* or *in vivo*. Moreover, a nucleic acid programmable protein array (NAPPA) has been developed that substitutes the printing of proteins on the array with printing cDNA that encode for the actual protein. The array is comprised of DNA that can be converted into protein with the addition of cell-free protein synthesis machinery. This approach represents a novel technique for studying protein interactions and disease biomarkers, because the proteins are made just in time for the assay without the need of purification.

In addition, the Medgene database, described by Dr. LaBaer, correlates genes to their particular disease. The Medgene database was able to find 2,100 genes related to breast cancer, many more than other databases.

Target Identification in Prostate Cancer

Michael McClelland, PhD, of the Sidney Kimmel Cancer Center, focused on genes associated with relapse in prostate cancer. Prostate cancer affects 1 in every 5 men living in North America and Europe. While diagnosis is easy, prognosis is not. By examining tumor samples after radical prostatectomy, it has been concluded that more than 2/3 of patients diagnosed do not need such treatment. The problem, therefore, rests with identifying those patients who need treatment from those that do not.

Samples of prostate tumors, taken from 750 patients, identified 1,100 genes associated with relapse, from which 144 genes specific to the stroma were predictive of relapse. Another prognostic method deals with a DNA methylation profile. DNA methylation is associated with cancer progression, and may be an imprint for past expression. To date, more than 50 novel differentially methylated genes in prostate cancer cells have been identified.

DIAGNOSIS, PROGNOSIS AND DISEASE MONITORING

Molecular Portraits by Cancer Type

Patrick Brown, MD, PhD, of Stanford University Medical School (Stanford, CA), compared cancer to an ecosystem. Just as tissue from different organs has a distinctive gene expression pattern, no two patients' malignant tissue is identical.

Because specific genes are expressed exclusively in different types of malignancy, the creation of molecular portraits for the various cancer types will provide the basis for diagnosis, early detection, imaging, targeted therapy, and discovering principles of pathophysiology. Researchers must also compare cancer samples to that of normal tissue samples from all parts of the body to discover meaningful differences in genetic expression and 'cross specificity' i.e., if cancer genes are expressed differently in the heart or ear.

The precise structure and physiology of human tissue depends on continuous communication and coordination among diverse cells. The body itself has thousands of cell signaling molecules. What must not be forgotten is that solid tumors are complex multicellular tissues whose stromal and vascular structure proliferate along with the malignancy.

Serum-based Approaches

According to Dr. Brown, serum is a 'magical' medium because human cells will not grow in a petri dish unless it is available. Besides promoting cell proliferation, serum also carries the physiologic signal that alerts the body to a wound. Serum causes cell migration and angiogenesis, inducing the clotting process that releases a signaling cascade to restore tissue. In essence, serum 'tricks' cells to think an emergency has occurred.

In the same way, cancer causes significant cellular events such as epithelial and stromal cells migration, extracellular matrix (ECM) and connective tissue invasion and remodeling, and new blood vessel recruitment. In an attempt to identify a serum wound response signature, 500 genes were identified. Applying this wound response signature to certain types of tumors has allowed for the identification of patients at an increased risk for metastasis and death. In particular, the wound response signature predicts a 3- to 4-fold higher mortality rate over 12 years in patients with early stage breast cancer. Dr. Brown is currently investigating how cancer cells falsely induce a wound response.

Samir Hanash, MD, PhD, of the Fred Hutchinson Cancer Research Center (Seattle, WA), addressed the potential of profiling serum to detect cancer. Different approaches to marker discovery include differentially expressed genes/proteins in tumor cells, proteins in biologic fluids, harnessing the immune response to detect tumor antigens, and serum profiling. The ultimate goal for the proteomic profiling of serum is to have a test for cancer just as the one for HIV.

The Human Proteome Organization (HUPO) Plasma Proteome Project sought to identify all the proteins present in human serum and plasma, while comparing the efficacy of various technologies for such a characterization. In total, HUPO identified 3,020 proteins, but more importantly, proteins associated with cancer were also found in the serum. An experiment was then devised to implant human cancer cells into a mouse, with the aim of detecting human tumor proteins in murine plasma. Results revealed that virtually all protein changes came from the mouse; a host response to the tumor occurred, but interestingly, there were some human proteins identified. Cystatin A (CSTA) was identified, and after further analysis, it was concluded that it was from the human cancer cell. Thus an *in vivo* model was able to prove that cancer proteins could be detected in the serum. Another protein from the human cancer cell, fatty acid binding protein (FABP), was also present in the murine serum.

According to Dr. Hanash, arrays can be established by fractionation of tumor proteins, that are specific for any type of cancer. When serum is added to these 'chips', a spot appears where hybridization occurs; thus, high spot expression indicates if a patient has cancer. A chip for lung cancer, for example, will reveal few/no spots when serum is taken from a patient with colorectal cancer. Furthermore, mass spectrometry may be performed on the spots to identify the specific type of protein involved in the malignancy. Future goals are to identify 10 to 20 of the most common proteins in colon cancer, followed by identifying those for lung cancer, ovarian cancer, etc. Over time, these chips can be combined to form a 'superchip' to diagnose any type of cancer a patient has by simply taking a serum sample. Since tumors have distinct expression, this technology has great potential.

Tumor Hypoxia

Another predictive model deals with hypoxia, a condition of low oxygen associated with the environment inside tumors. A molecular signature for hypoxia may forecast recurrence and metastasis in patients with breast cancer. Patients expressing genes associated with this model were twice as likely to die from the disease over the course of 12 years when compared to patients without the signature. When compared to other prognostic parameters such as tumor grade, size, location, previous chemotherapy status, etc., both the hypoxia and wound response signatures were significantly and individually the most important parameters in breast cancer prognosis.

Stem Cells

Gennadi Glinsky, MD, PhD, of the Sidney Kimmel Cancer Center, discussed the prognostic value of stem cells in multiple types of cancer. The 11-gene bmi-1 pathway is essential for self-renewal of normal stem cells. In transformed cells, however, activation of stem cell pathways may contribute to tumor progression and metastasis. Stem cells can fuse to cancer cells, thereby forming a hybrid that induces self-renewing cancer stem cells that are precursors to metastasis. *In vivo*, increased expression of bmi-1 genes is associated with prostate cancer progression. In 9 metastatic and 23 primary human prostate tumor samples, the bmi-1 pathway consistently displayed a stem cell-resembling expression profile. Expanding on this data, tumor samples from patients with prostate cancer (n=167), breast cancer (n=256), lung cancer (n=211), ovarian cancer (n=50), bladder cancer (n=31), gastric cancer (n=89), lymphoma (n=390), mesothelioma (n=17), medulloblastoma (n=60), glioma (n=50), and AML (n=401) were analyzed. The bmi-1 pathway signature proved to be a powerful predictor of short interval to disease recurrence, distant metastasis, and death in 10 types of cancer. In conclusion, a conserved bmi-1 oncogene-driven pathway, expressed in normal stem cells and highly malignant cancer types, represents a prominent drive toward metastatic disease accompanied with an unfavorable prognosis.

MALDI (Matrix Assisted Laser Desorption Ionization) Mass Spectrometry

David Carbone, MD, PhD, of Vanderbilt University Medical Center (Nashville, TN), discussed the applications of mass spectrometry for the prognosis of lung cancer. This year has marked a milestone in cancer. For the first time, more people in the USA have died from cancer than from heart disease. In particular, lung cancer is the number one cause of cancer-related deaths. Mass spectrometry could prove to be a useful device to establish disease markers, because unlike RNA-based methods, it is closely associated with protein expression.

The MALDI mass spectrometer measures the time it takes for a laser beam to travel through a tissue sample. In this way, different mass spectra are obtained for normal and cancer tissue. A comparative analysis has yielded 25 unique signals able to distinguish primary non-small cell lung cancer (nsclc) from metastatic nsclc. Moreover, 15 proteins discovered through this method can predict patient mortality at 1 year after disease onset. Among these 15 proteins are thymosin-β 4 and ubiquitin (Ub), which are downregulated in mouse tumors after erlotinib (Tarceva; OSI Pharmaceuticals) treatment. While some mass spectrometry peaks are validated by immunohistochemistry, others are not. Moreover, serum from patients can also be subjected to mass spectrometry.

Unlike prostate specific antigen (PSA) used in the screening of prostate cancer, there is no single marker for lung cancer. This lack of a diagnostic tool leads to mismanagement of the disease, as is evident by the fact that

20% of patients who have a lung removed are found to have no cancer after surgery. Serum profiling in lung cancer has revealed 57 distinct serum features, with 99% specificity. Further refinement of this paradigm will include samples from 508 patients with cancer. In an international clinical trial that administered gefitinib (Iressa; AstraZeneca) to patients with lung cancer, 34 peaks yielded a clear profile for identifying those patients who responded to therapy, with 75% accuracy.

Unlike a primary biopsy, serum is easy to obtain, making it an ideal diagnostic medium. A new clinical trial to enroll 200 patients is now planned to further evaluate this mass spectra profile.

MALDI may be useful for profiling tumors and establishing a means for early cancer detection, prognosis, and diagnosis. Mass spectrometry is a simple, fast, and inexpensive test that preserves tissue architecture, but is limited to highly abundant low molecular weight proteins, desorption (suppression) by high abundant proteins like albumin, and the inability to use formalin-fixed tissues.

Dr. Carbone also highlighted exciting advancements in imaging techniques based on mass spectrometry, which can be performed on arrays, thereby generating 2-dimensional measurements, without the aid of an antibody or requirement for knowing the protein being investigated. A technique has also been developed to image the entire body of a rat. In this case, according to Dr. Carbone, a small peak, though seemingly insignificant, directly related to protein expression in the brain. Other individual peaks perfectly highlighted the heart, lungs, and other organs. Therefore, novel future applications of mass spectrometry may include the ability to visualize how drugs are distributed throughout the body.

MECHANISMS IN MALIGNANCY

THE C-SRC SIGNAL TRANSACTION PATHWAY

Aberrations in signaling pathways that regulate normal cell growth, activity, and function, are a hallmark of malignancy. Among key signaling proteins/enzymes are kinases that control many properties of cells, including growth, development, and survival. Abnormal activation of oncogenic kinases may lead to uncontrolled cell proliferation and cancer. These proteins represent highly attractive, well defined targets for cancer therapy. One such signaling molecule is c-Src, which functions at the hub of a vast array of signal transduction cascades that influence cellular proliferation, differentiation, motility, and survival.

Success of imatinib mesylate (Gleevec, Glivec; Novartis), a small molecule inhibitor of the Bcr-Abl chimeric gene product implicated in chronic myelogenous leukemia (CML) that has also proven effective against such solid malignancies as gastrointestinal stromal tumors (GIST) and is being evaluated in many others, encouraged investigators to pursue research in kinase inhibitors participating in various signaling pathways associated with human malignancy. Also, the emergence of imatinib-resistant phenotypes has created a need for either more effective drugs, or follow-on agents to treat imatinib failures. Src is one such target being evaluated in both of these cases. Also, dual Src/Abl inhibitors may avoid resistance to imatinib generated by mutated forms of the Bcr-Abl kinase in patients with CML (Druker BJ, etal, Cancer Res, 15 Dec 2002; 62:7149-53).

The immediate main objective of drug development programs in this area is to introduce a viable competitive/synergistic agent to imatinib, to treat all phases of CML. CML is a malignancy that starts in the bone marrow and invades the blood. The worldwide incidence of CML is 1 to 2 cases per 100,000 per year. CML is responsible for 15% to 20% of all adult cases of leukemia. In the USA, the incidence of CML is 1.6 per 100,000 per year. The American Cancer Society (ACS) estimates that 4,600 cases were diagnosed in the USA in 2004. It is estimated that more than 16,000 patients in the USA are currently living with CML, which usually occurs in middle-aged adults; the average age at onset is 50 years. The commercial opportunity associated with this rather rare malignancy underscores the power of chronic treatment in oncology. Global sales of imatinib in 2004 topped $1,634 million. The rapid penetration of this drug globally is illustrated by the fact that USA sales are $368 million compared to $1,266 million in the rest of the world. The main reason for the large revenues is the fact that imatinib is a chronic treatment for a disease that is amenable to maintenance therapy. CML is a paradigm of the commercial opportunity in transforming cancer from a short term disease into a chronic condition managed by drug therapy.

Among the many sources reviewed for this article, these two reviews have been particularly helpful:

Ishizawar R, and Parsons SJ, *c-Src and cooperating partners in human cancer*, Cancer Cell, Sep 2004;6(3):209-14

Dehm SM, and Bonham K, *SRC gene expression in human cancer: the role of transcriptional activation,* Biochem Cell Biol, Apr 2004;82(2):263-74

THE SRC KINASE FAMILY (SKF)

The viral src gene, originally discovered by its homology to the Rous sarcoma virus oncogene protein pp60(v-Src), was the first retroviral oncogene to be identified, and its cellular counterpart was the first proto-oncogene to be discovered in the vertebrate genome. Subsequently, Src became important as an entry point into the molecular genetics of cancer (Martin GS, Nat Rev Mol Cell Biol, Jun 2001;2(6):467-75).

The Src kinase family (SKF) on nonreceptor protein tyrosine kinases (NRPTK) consisting of members Src, Yes, Fgr, Yrk, Fyn, Lyn, Hck, Lck, and Blk, represents one of the richest and most diverse enzyme families in terms of drug targets. From the N to C termini, Src kinase family members

comprise Src homology (SH) domains SH4, SH3, and SH2, a linker region, a catalytic domain (SH1), and the C-terminal 'tail' sequences. SH3 domain and C-terminal sequences are required for full regulation of c-Src activity. Sequences between the SH4 and the SH3 domains vary considerably among different members of the Src family.

SKF is an intermediary of information transfer, controlling such diverse processes as cell division, motility, adhesion, genome maintenance, and survival. Src kinases interact with a variety of cell-surface receptors, and participate in various intracellular signal transduction pathways. SFK are activated by G-protein-coupled receptors (GPCr). SFK are also important modulators of T-cell activation. SFK members are being pursued as targets for indications that include cancer, osteoporosis, cardiovascular and autoimmune diseases, viral infections, and graft rejection after organ transplantation.

In 1991, investigators at the University of Helsinki, in Finland, isolated a cDNA encoding a novel NRPTK family member, named cyl (consensus tyrosine-lacking kinase), from the K562 human leukemia cell line. The cloned novel cytoplasmic tyrosine kinase lacking the conserved tyrosine autophosphorylation site (Y416src) was designated c-Src tyrosine kinase (Csk). Csk that contains features of known cytoplasmic tyrosine kinases, including amino-terminal SH3 and SH2 domains, downregulates tyrosine kinase activity of the c-Src oncoprotein through tyrosine phosphorylation of the c-Src carboxy terminus. Therefore, Csk acts as a negative regulatory site, potentially functioning as an antioncogene (Partanen J, etal, Oncogene, Nov 1991;6(11):2013-8). Subsequently, these investigators mapped the Csk gene to 15q23-q25 by *in situ* hybridization (Armstrong E, etal, Cytogenet Cell Genet 1992;60(2):119-20).

C-SRC

Human pp60c-Src, usually referred to as c-Src (Src), is a 60 kDa nonreceptor tyrosine kinase encoded by the src gene, mapped to 20q12-q13. It is the cellular homolog to the potent transforming v-src viral oncogene. The 3-dimensional structure of c-Src was delineated by investigators at Children's Hospital (Boston, MA) (Xu W, etal, Nature, 13 Feb 1997;385(6617):595-602; comment:582-3, 585).

Src kinase is a signal transduction modulator, and a critical component of many of the key signaling pathways currently thought to be pivotal in carcinogenesis. In contrast to its highly regulated role in normal cells, Src kinase is deregulated with significantly increased activity in many human malignancies. Src kinase has been implicated in a number of processes important for malignant growth, including growth factor-driven cell proliferation, VEGF-dependent angiogenesis, and metastasis.

In cells, endogenous Src is switched from an inactive to an active state by a variety of mechanisms that simultaneously relieve constraints on the kinase and protein-interacting Src homology (SH) domains. As a result, Src moves to the cell periphery, often to sites of cell adhesion, where myristoylation mediates its attachment to the inner surface of the plasma membrane. From these peripheral sites, Src's catalytic activity initiates intracellular signal transduction pathways that influence cell growth and adhesion that contribute to control of cell migration. Deregulation of Src in cancer cells may enhance tumor growth and/or stimulate relatively nonmotile cells to become migratory or invasive. Src may also influence the life or death decisions that cells make during many biologic processes and, therefore, Src modulation in cancer cells can alter cell responses that are often perturbed in cancer (Frame MC, Biochim Biophys Acta, 21 Jun 2002;1602(2):114-30).

Src plays key roles in signal transduction following growth factor stimulation and integrin-mediated cell-substrate adhesion. Src transduces signals controlling cellular processes such as motility, adhesion, invasion, proliferation, differentiation, and survival. In normal cells, Src has been implicated in the control of cell division, the production of autocrine growth factors, the cell's survival response, as well as in cell motility. Src kinase activity is necessary for cells to enter the cell cycle when exposed to mitogens such as platelet-derived growth factor (PDGF). Src activity initiates a signal transduction cascade, involving the adaptor protein Shc, which culminates in the transcriptional activation of the transcription factor Myc. Furthermore, this requirement for Src is abrogated in cells lacking the tumor suppressor p53, suggesting that another of Src's functions in normal cells is to suppress the actions of p53 (Courtneidge SA, Biochem Soc Trans, Apr 2002;30(2):11-7).

Src kinase is also highly expressed in osteoclasts. Targeted disruption of the src gene in mice induces osteopetrosis, i.e., partial filling of the bone marrow cavity attributable to impaired bone resorption, rising from the inability of osteoclasts to migrate and bind to the bone surface; functions essential for bone resorption. In the osteolytic bony metastases characteristic of late stage breast cancer and myeloma, osteoclast and tumor cell activities are linked in a 'vicious cycle', in which tumor cells stimulate osteoclast bone resorption, which releases growth factors locally, further stimulating tumor growth within the bone.

Post-translational modification and negative regulation of c-Src activity is the result of phosphorylation of a tyrosine residue located at the C-terminus of the protein by Csk. Inactivation of Csk in mice led to an order of magnitude increase in activity of Src and related Fyn kinases, indicating that Src family kinase activity is critically dependent on phosphorylation by Csk (Imamoto A and Soriano P, Cell, 18 Jun 1993;73(6):1117-24).

The major consequence of increased c-Src activity is to promote an invasive tumor phenotype characterized by breakdown of cell-cell adhesion, increased adhesion of cells to the matrix, and formation of focal adhesions and podosomes. Src kinase activity is required for adhesion turnover associated with cell migration in cancer cells and, in addition to its catalytic activity, Src also acts as an adap-

tor to recruit other kinases that can phosphorylate key substrates (Brunton VG, etal, Cancer Res, 15 Feb 2005;65(4):1335-42). Accordingly, inhibition of c-Src kinase activity in preclinical models restores cell-cell adhesion, inhibits cell migration and invasion, and reverses the Src modulated invasive phenotype.

Numerous signal transaction pathways are mediated by c-Src. Receptor tyrosine kinases (RTK) that activate s-Src include platelet-derived growth factor receptor (PDGFr), epidermal growth factor receptor (EGFr), and various integrins, among others. Evidence to date supports a model wherein c-Src potentiates survival, proliferation, and tumorigenesis of EGFr family members, in part by associating with them. Phosphorylation of EGFr by c-Src is also critical for mitogenic signaling initiated by EGFr itself, as well as by several GPCr, a cytokine receptor, and the estrogen receptor (Er). Thus, c-Src appears to have pleiotropic effects on cancer cells by modulating the action of multiple growth-promoting receptors (Biscardi JS, etal, Breast Cancer Res 2000;2(3):203-10).

The Src downstream pathway includes the phosphatidylinositol 3'kinase (PI3K) and p42 (ERK2)/p44 (ERK1) MAPK cascades, culminating in the AP-1/LEF-1 response elements of the matrix metalloproteinase matrilysin (MMP-7) promoter implicated in invasion and metastasis.

Because of c-Src's involvement in the malignant phenotype, a major effort was undertaken to understand the mechanisms of c-Src activation in human cancer. Originally it was thought that mutations in c-Src were responsible for it being implicated in carcinogenesis and metastasis, based on the finding that a constitutively active form of v-Src kinase, encoded by the mutant Src gene within the Rous sarcoma virus genome, can transform fibroblasts. However, it was subsequently shown that c-Src was rarely mutated in human malignancies.

Although evidence for activating mutations within the cellular Src homolog, c-Src, in human tumors is limited and controversial, there is a significant body of data describing high levels of c-Src kinase expression and activity in many human tumor types. In many cases, this appears to be driven by post-translational activation mechanisms. Src expression has been linked to poor clinical prognosis, tumor progression, and in particular, tumor dissemination. These observations are supported by accumulating preclinical data suggesting that increased Src kinase activity predominantly leads to changes in cell behavior that contributes to an invasive tumor phenotype (Green T, etal, AACR05).

Src is overexpressed and highly activated in a wide variety of human malignancies, and also appears to correlate with disease stage. Src activation has been documented in upwards of 50% of tumors derived from the colon, liver, lung, breast, and pancreas. There appears to be a significant relationship between Src activation and cancer progression, and Src may influence development of the metastatic phenotype. Therefore, Src may play a role as a critical component of the signal transduction pathways that control cancer cell development and growth, making it a target in drug discovery efforts.

Interestingly, it was reported that an Src mutation was observed in a small subset of advanced colon tumors. In addition, elevated src transcription has been identified as yet another mechanism contributing significantly to c-Src activation in a subset of human colon cancer cell lines. Also, it has been shown that histone deacetylase (HDAC) inhibitors, agents with well documented anticancer activity, repress src transcription in a wide variety of human cancer cell lines (Dehm SM and Bonham K, Biochem Cell Biol, Apr 2004;82(2):263-74).

Also, Src has a similar structure as Abl, the target of imatinib. Therefore, inhibitors of Src may also inhibit Abl.

Bone Metabolism

The role of Src in bone metabolism, first observed in Src-deficient mice, has since been confirmed using low molecular weight Src inhibitors in animal models of osteoporosis (Susva M, etal, Trends Pharmacol Sci, Dec 2000;21(12):489-95). Malignancies such as breast, lung, and prostate cancer, commonly metastasize to bone, and may also cause humorally mediated hypercalcemia by producing factors that stimulate bone resorption systemically in the absence of bone metastasis. Metastatic tumor cells promote osteolysis by releasing paracrine factors in the bone microenvironment, resulting in an indirect stimulatory effect on osteoclasts, the principal bone resorbing cells.

Osteoclasts must express Src to resorb bone normally, as evidenced by the abnormal cellular morphology and impaired functional properties of osteoclasts in src -/- mice associated with the development of osteopetrosis. According to investigators at Washington University School of Medicine (Saint Louis, MO), mice with homozygous germ line deletions of the src proto-oncogene have defective osteoclasts that fail to resorb bone resulting in severe osteopetrosis. Owing to their defective osteoclast function, it was predicted that src-/- mice would be protected from bone metastases. Although 6/7 src-/- mice injected with B16 cells developed widespread visceral metastases with bone tumors present in 6/7 src-/- mice, on histologic examination, tumors were present in the bone marrow space, but no bone destruction was evident. In contrast, among the 4/4 wild type mice that developed visceral metastases and the 3/4 with bone tumors, histologic examination revealed extensive bone destruction associated with tumors in the bone marrow space. Thus, src-/- mice were protected from bone destruction associated with bone metastases, suggesting that functional host osteoclasts are critical for bone destruction associated with metastasis. Therefore, specific inhibition of host Src tyrosine kinase activity may represent a therapeutic target in the treatment of osteolytic bone metastasis (Nestor NJ, etal, AACR03, Abs. 3425).

Regulation of Angiogenesis

Gary E. Gallick, PhD, and colleagues at M. D. Anderson Cancer Center (Houston, TX) hypothesized that because Src has been implicated in both the regulation of expression and response to proangiogenic factors, it would be a critical mediator of angiogenesis induced by colorectal carcinoma cells. To examine Src-mediated angiogenic properties, immortalized endothelial cells from both the colon (CEC) and liver (HEC) were stimulated with conditioned media from HT29 human colorectal carcinoma cells previously treated with a selective Src kinase inhibitor such as pyrazolopyrimidine 2 (PP2) or dimethyl sulfoxide (DMSO) control.

Conditioned media from HT29 colorectal carcinoma cells induced a ~2-fold increase in proliferation and migration in CEC, and >3-fold increase in HEC. Conditioned media from the Src-inhibited colorectal carcinoma cells failed to induce significant proliferation or migration in either cell line. Likewise, EGF, basic fibroblast growth factor (bFGF), VEGF-A, and hepatocyte growth factor (HGF), induced a 5.0-, 3.7-, 4.4-, and 3.9-fold increase in migration in HEC, respectively, and a 3.3-, 2.4-, 2.7-, and 4.0-fold increase in migration in CEC, respectively. In contrast, while EGF and bFGF caused ~2-fold increase in proliferation in both HEC and CEC, VEGF failed to increase proliferation above control values. HGF induced a 1.5-fold increase in CEC proliferation, but failed to induce proliferation in HEC. Pretreatment with the selective Src inhibitor abrogated endothelial proliferation and migration induced by all factors. EGF treatment of HEC induced a 2-fold increase in Src activity, a >5-fold increase in phosphorylated Erk, and a ~3-fold increase in phosphorylated Akt, all of which were effectively blocked by Src inhibition. Therefore, angiogenesis induced by colorectal carcinoma cells is regulated by Src kinase (Lesslie DP III, etal, AACR05, Abs. 2024).

Dr. Gallick and colleagues also examined the role of Src, frequently activated in pancreatic ductal adenocarcinoma (PDAC), in regulating interleukin (IL)-8 and VEGF expression. Both IL-8 and VEGF are angiogenic peptides that contribute to progression of PDAC. In addition, the role of Src-mediated signaling pathway was examined that contributes to angiogenesis *in vivo*. Scr inhibitors PP2 or AP23846 (an analog of AP23464) were added to cells for selective inhibition of SFK that resulted in significant dose-dependent decreases in IL-8 and VEGF expression. Infiltration of vessels from PDAC cells was nearly abrogated when exposed to conditioned media from cells treated with either PP2 or AP23846 (Trevino JG, etal, ASCOGI05, Abs. 152).

Promotion of Metastasis

Src plays a critical role in tumor-cell migration and invasion, and is involved in controlling contacts between cells that maintain the integrity of normal tissues. Abnormal Src activity in tumor cells leads to the breakdown of these contacts, allowing cancer cells to spread to distant sites and establish tumor colonies. Inhibitors of Src block signaling pathways that control migration of cancer cells, and may prove an effective approach for the prevention of metastatic spread of solid tumors from primary to distant sites.

Involvement of Src kinase during metastasis was explored using the NBT-II rat bladder carcinoma cell line, which can be induced to scatter *in vitro* by Src kinase. Using NBT-II cells overexpressing a dominant-negative mutant of Src (srcK-) or Csk, the natural inhibitor of Src, it was demonstrated that although Src activity is not required for growth of tumors derived from NBT-II cells injected into nude mice, it is crucial for the emergence of micrometastases (Oncogene 2002; 21: 2347-2356).

MALIGNANCIES ASSOCIATED WITH C-SRC

Overexpression and/or hyperactivation of c-Src kinase are common features of many human malignancies including brain, bone, breast, colon, ovarian, pancreatic, and prostate cancer, and leukemia. Therefore, c-Src is a target with potential broad applications in neoplastic disease.

Bone Cancer

Osteoclasts express Src in order to resorb bone normally, as evidenced by the abnormal cellular morphology and impaired functional properties of osteoclasts associated with the development of osteopetrosis in src -/- mice. However, although Src expression is ubiquitous, in the src -/- mice the only phenotype observed is in bone. This fact renders Src as an excellent target for treatment of osteolytic bone metastasis from such malignancies as breast, lung, and prostate cancer that commonly metastasize to bone. Even when not metastasized to bone, these malignancies induce humorally mediated hypercalcemia by producing factors that stimulate bone resorption. Also, metastatic tumor cells promote osteolysis around them by releasing paracrine factors in the bone microenvironment, resulting in an indirect stimulatory effect on osteoclasts (Shakespeare W, etal, AACR03, Abs. 3971).

Breast Cancer

Overexpression or increased activity of c-Src is frequently detected in human breast cancer, implicating involvement of c-Src in the etiology of this malignancy. However, overexpression of c-Src in tissue culture cells resulted in a weakly or nontransforming phenotype, indicating that Src alone is not sufficient for oncogenesis. Rather, c-Src appears to be required to maintain the oncogenic phenotype of breast cancer cells (Ishizawar RC, etal, J Biol Chem, 28 May 2004;279(22):23773-81). For instance, Src appears to potentiate mitogenic signals from transmembrane receptors. Evidence from murine fibroblast models and human breast cancer cells indicates that c-Src and human EGFr synergize to enhance neoplastic growth of mammary epithelial cells (Slack JK, etal, Oncogene, 22 Mar 2001;20(12):1465-75). Src and members of the EGFr family are simultaneously overexpressed in ~70% of cases of breast cancer.

When SFK expression was measured in 52 human mammary tumor specimens, compared with normal tissue from the same patient, the mean enzymatic activity was moderately elevated in the tumor specimens, with 25 tumor samples having higher activity than the corresponding normal tissue, and 17 having lower activity; no activity was detected in 10 tumor/normal pairs (Reissig D, etal, J Cancer Res Clin Oncol, Apr 2001;127(4):226-30).

Src may play a role in breast cancer via its interaction with steroid hormone receptors. Estrogens affect cellular processes by binding to their cognate receptors Erα and Erβ, playing a key role in breast cancer. Investigators examined the role of the K303r Erα mutant receptor and c-Src kinase in promoting breast tumor growth and metastasis. The K303R Erα mutation is present in about one third of hyperplastic and invasive breast lesions. Additionally, cells expressing this mutant Erα are more sensitivity to subphysiologic estrogen concentrations, resulting in increased Erα-induced transactivation and cellular proliferation. Treatment of MCF-7 cells expressing wild type and mutant Erα with AZD0530 abolished c-Src tyrosine phosphorylation, and was effective at reducing basal and estrogen-induced invasion together with cell growth, suggesting a role for c-Src in cell proliferation and estrogen-mediated invasion in breast cancer cells (Herynk M, etal, AACR05, Abs. 264).

Chronic Myelogenous Leukemia (CML)

CML is the poster child of the potential of oncogenic protein inhibition in cancer. Bcr-Abl nonreceptor protein kinase, the hallmark of CML, is the target of imatinib, one of the few indisputable successes in the treatment of cancer. Almost 96% of patients with CML respond to imatinib. However, imatinib resistance occurs in patients with CML in all phases of the disease through mechanisms that are only partially understood. Point mutations within the Abl kinase and SH3 domain emerged as a major mechanism of imatinib resistance.

Point mutations and amplification of the bcr-abl gene detected in patients with resistant disease alone do not account for resistance in all patients. A role of SKF members has been described in CML, particularly in resistant disease. Imatinib-resistant CML cell models and analysis of clinical specimens suggest that activation of hematopoietic cell-type specific SFK such as Lyn and Hck that are not inhibited by imatinib, reduce imatinib-mediated antileukemic activity, and play a role in resistance.

Based on animal models of CML, it appears that both Bcr-Abl and SFK inhibition are necessary to overcome imatinib resistance *in vivo*. In imatinib-sensitive CML cells, imatinib reduced Bcr-abl and specific phosphorylation of CrkL, a substrate of Bcr-Abl, and coordinately reduced activation of SFK, suggesting upstream regulation of the SFK by Bcr-Abl in imatinib-responsive cells. However in patients with imatinib-resistant CML who do not lose sensitivity to ima-tinib through Bcr-Abl mutations, imatinib failed to suppress SFK, suggesting SFK regulation by Bcr-Abl independent mechanisms. Bcr-Abl/SFK complexes were detected in imatinib sensitive, but not resistant cells. Autonomous regulation of SFK in imatinib-resistant CML specimens and cell survival could be overcome with BMS-354825, a novel Src/Abl dual kinase inhibitor. Targeted interference of SFK expression could also be overcome by siRNA. Together, these results suggest that SFK play a role in imatinib resistance. Therefore, inhibition of both Bcr-Abl and SFK may circumvent the development of imatinib resistance, and increase the clinical activity of kinase-targeting therapies in CML (Donato NJ, etal, AACR05, Abs. 1507).

Colorectal Cancer

Colorectal cancer was the first human cancer to be associated with c-Src. Overexpression or increased activity of Src has been reported in >70% of human colon tumors. Also, blocking Src in human colon tumor xenografts prevents tumor growth.

Enhanced expression of Src plays an important role in the spread of colon cancer, specifically disrupting proper assembly of E-cadherin-dependent cell-cell contacts leading to cancer-cell migration (Frame MC, Biochim Biophys Acta, 21 Jun 2002;1602(2):114-30, and Frame MC, etal, Nature Cell Biology 2002 4:632).

Elevated levels of both c-Src protein and mRNA in a subset of human colon cancer cell lines were attributable to increased transcription of the src gene, and correlated strongly with increased c-Src kinase activity. Decreased c-Src mRNA stability was also observed in cell lines that displayed src transcriptional activation. These findings provide evidence that src transcriptional activation is an important determinant of c-Src expression and activity in colon cancer cell lines (Dehm S, etal, FEBS Lett, 5 Jan 2001;487(3):367-71).

Ovarian Cancer

Alteration of c-Src activity greatly influences the tumorigenic potential of metastatic ovarian cancer cells. Src associates tightly with members of the EGFr family, and in particular, with HER-2/neu, and plays a major role in modulating the expression and secretion of invasive or angiogenic factors from metastatic ovarian cancer cells. Src is overexpressed and activated in a majority of late stage ovarian cancer, as well as in a panel of cultured malignant human ovarian epithelium grown *in vitro*, but not in normal ovarian epithelium or immortalized normal ovarian epithelium. Although Src overexpression was found to be frequently, but not always, associated with HER-2/neu overexpression, no statistical association between Src and Her-2/neu overexpression could be demonstrated (Wiener JR, etal, Gynecol Oncol, Jan 2003;88(1):73–79).

Investigators at the University of Miami observed that both the hyaluronan (HA) receptor, CD44, and c-Src kinase are expressed in the human ovarian tumor cell line

SK-OV-3.ipl, and that these two proteins are physically associated as a complex *in vivo*. Furthermore, binding of HA to SK-OV-3.ipl cells promotes c-Src kinase recruitment to CD44, and stimulates c-Src kinase activity, which, in turn, increases tyrosine phosphorylation of the cytoskeletal protein, cortactin. Subsequently, tyrosine phosphorylation of cortactin attenuates its ability to cross-link filamentous actin *in vitro*. In addition, transfection of SK-OV-3.ipl cells with a dominant active form of c-Src (Y527F) cDNA promotes CD44 and c-Src association with cortactin in membrane projections, and stimulates HA-dependent/CD44-specific ovarian tumor-cell migration. Overexpression of a dominant-negative mutant of c-Src kinase (K295R) in SK-OV-3.ipl cells impairs the tumor cell-specific phenotype. Taken together, these findings strongly suggest that CD44 interaction with c-Src kinase plays a pivotal role in initiating cortactin-regulated cytoskeleton function and HA-dependent tumor cell migration, which may be required for human ovarian cancer progression (Bourguignon LY, etal, J Biol Chem, 9 Mar 2001;276(10):7327-36).

Pancreatic Cancer

Src is aberrantly activated and/or overexpressed in pancreatic tumors. Src expression and activation correlates with VEGF production in several pancreatic cancer cell lines. Furthermore, pharmacologic inhibition of SFK activity by PP2, significantly decreased both constitutive VEGF production and EGF-induced VEGF production. These results suggest that SFK regulate VEGF expression. To examine the specific role of Src in VEGF expression, stable clones of L3.6pl PDAC cells were established, in which Src expression was reduced by siRNA. Expression of VEGF was reduced in these clones at levels similar to those achieved with PP2. Upon EGF stimulation, both the PI3K/Akt and p38 mitogen activated protein kinase (MAPK) pathways important for EGF-mediated VEGF production in PDAC cells were activated in an SFK-dependent fashion. Also, media from Src-inhibited L3.6pl cells failed to promote angiogenesis into gel-foams subcutaneously (SC) implanted into mice, whereas media from control cells promoted a readily detectable angiogenic response. Results suggest that SFK inhibitors may be important therapeutic agents in PDAC (Summy JM, etal, AACR05, Abs. 4556).

IL-8 is a proangiogenic factor commonly secreted by PDAC. Src correlates with IL-8 production. Activated Src increased by ectopic expression in PANC-1 cells with low endogenous Src activity, results in a significantly increase of IL-8 production. In contrast, inhibition of endogenous SFK activity with pharmacologic inhibitors, or by decreasing c-Src specifically by stable siRNA expression in L3.6pl cells with high Src expression and activity, causes significant decreases in IL-8 production. Src mediates IL-8 expression by regulating critical 'downstream' signaling pathways in PDAC cells (Trevino JG Jr, etal, AACR05, Abs. 4557).

Prostate Cancer

It appears that Src activation is important in prostate tumor progression. Investigators at M. D. Anderson Cancer Center, under a grant by the Department of Defense (DoD), demonstrated that increased Src activity leads to increased expression of VEGF, implicating Src in promotion of angiogenesis by prostate tumor cells. Also increased Src expression was linked to increased tumorigenic growth in nude mice.

Src may also act via the androgen receptor (Ar). Treatment of human prostate carcinoma-derived LNCaP cells with androgen, or estradiol, triggers simultaneous association of Ar and estradiol receptor ß with Src, activating the Src/Raf-1/Erk-2 pathway, and stimulating cell proliferation. Src SH2 interacts with phosphotyrosine 537 of the estradiol receptor, and the Src SH3 domain with a proline-rich stretch of Ar.

AGENTS IN DEVELOPMENT TARGETING THE C-SRC PATHWAY

Located intracellularly in the cytoplasm, c-Src has proven an attractive target for orally available small molecule drugs. SKF are critical participants in many signaling pathways that regulate all stages of malignancy, from oncogenesis to metastasis, in many cell types. Therefore, SKF, and particularly Src modulators, are expected to have a role to play, alone or in combination with other anticancer agents, in a broad spectrum of malignancies at all stages of the disease. However, this sector is in early stages of development, and still faces a lot of challenges both at the bench and the clinic.

One issue in drug development in this area is specificity. Ideally, specificity is not only desirable, but a requirement to ensure safety and reliability. However, it may also limit the effectiveness or use of the agent in development. For instance, use of imatinib has expanded when it was discovered that in addition to blocking Bcr-Abl, it also targeted c-Kit, making it an effective treatment in GIST. Subsequently, it was shown that imatinib also targets platelet-derived growth factor receptor alpha (PDGFrA), further expanding its potential indications. In contrast, gefitinib (Iressa; AstraZeneca), a small molecule ATP analog blocking the activity of EGFr, appears to work better in a small subset of non-small cell lung cancer (nsclc), with specific somatic mutations in the ATP binding pocket of EGFr that confer a heightened aggressiveness to the disease. Somatic mutations the EGFr gene were found in 15 of 58 unselected tumors from Japan, and 1 of 61 from the USA. This finding was quite remarkable, and explained why although treatment with gefitinib caused tumor regression in some patients with nsclc, the phenomenon was more frequently observed in Japan (Paez JG, etal, Science, 4 Jun 2004;304(5676):1497-500, and Lynch TJ, etal, NEJM, 20 May 2004;350(21):2129-39). Lack of broad activity in EGRr-overexpressing tumors has virtually destroyed gefitinib's opportunity as an anticancer agent. In this case, high selectivity was a drug's undoing.

In support of high selectivity in the design of Src inhibitors is the fact that Src belongs to a family whose other members participate in critical functions in normal cells. Therefore, highly specific inhibitors of c-Src need to be developed to avoid any untoward toxicity. However, broad spectrum Src inhibitors appear to be much more effective in treating imatinib-resistant disease arising from to mutations in the catalytic domain of Bcr-Abl (Shah NP, etal, Science, 16 July 2004;305 (5682);399-401). Therefore, it has been suggested that high selectivity may not be a good thing. Unfortunately, it appears that each case has to be evaluated in its own merits, and both high and low specificity inhibitors may have a role in the treatment of cancer that needs to be individually investigated in clinical trials.

Strategies to inhibit c-Src include blocking the protein's catalytic activity, blocking protein-protein interactions, and interfering with protein maturation or stability. A more indirect approach is to block/modulate other points in various pathways involving Src.

Drugs that inhibit protein kinases at their catalytic sites are ATP analogs such as imatinib. This approach has been the most prevalent basis for the development of small molecule inhibitors of protein kinases. ATP analogs, such as tyrophostins and pyrimidine compounds, directly inhibit tyrosine kinase activity of c-Src and/or related kinases. Investigators at Novartis (Basel, Switzerland) reported that optimized 5-aryl-pyrrolo[2,3-d]pyrimidines incorporating different N(7)-substituents are highly potent c-Src inhibitors. Some exhibited excellent specificity towards other receptor and nonreceptor tyrosine kinases and were characterized by a good PK profile (Altmann E, etal, Mini Rev Med Chem, Jun 2002;2(3):201-8).

Nonkinase inhibitors in development interact with the noncatalytic SH2 domain of Src, involved in protein-protein interactions that mediate signal transduction. SH2 domains mediate protein-protein interactions through short contiguous amino acid motifs containing phosphotyrosine. These domains are key regulators in a variety of cellular processes and, therefore, are attractive drug targets. However, development of small molecule inhibitors of protein-protein interactions is very challenging, because such targets are considered to be barely druggable.

A more general approach in blocking Src activity is by using agents that trigger protein instability or prevent maturation of newly synthesized proteins. One such class of drugs, are inhibitors of heat-shock protein 90 (Hsp90), a protein chaperone that guides the maturation of c-Src and other oncoproteins to a fully functional conformation and intracellular localization (Xu Y, etal, PNAS USA, 5 Jan 1999;96(1):109-14). Among HSP90 inhibitors are the natural products geldanamycin, herbimycin, and radicicol.

An even more indirect strategy in blocking Src activity is by modulating other factors associated with the proper functioning of Src. For instance, it may be possible to interfere with myristoylation that occurs during the protein maturation process. During myristoylation, a myristoyl moiety is added to the N terminus of Src, directing it to cellular membranes required to confer it functional activity. Elevated levels of N-myristoyltransferase, detected in some colon cancer cell lines and gallbladder tumors (Rajala RV, etal, Biochem Biophys Res Commun, 14 Jul 2000;273(3):1116-20), appear to correlate with poor prognosis. It may, therefore, be possible to inhibit c-Src and other myristoylated signaling proteins involved in cancer progression by targeting the enzyme that mediates myristoylation. Myristoylation is a basic process that confers functionality to proteins. It may be a bad thing when acting on oncogenic proteins, but it is vital in rendering functionality to tumor suppressor proteins.

Exhibit 1 describes several selective Src inhibitors in preclinical development and clinical trials. One of the first c-Src inhibitors was PP2, now used extensively in drug development.

AP22408, AP23236, and AP23451

Using structure-based design, scientists at Ariad Pharmaceuticals (Cambridge, MA) created a number of Src inhibitors targeted to the bone, including AP22408, AP23236, and AP23451. AP22408, a bicyclic nonpeptide inhibitor, was designed to mimic the pTyr structure of binding proteins in complex with the SH2 domain of c-Src (Shakespeare WC, etal, PNAS USA, 2000;97; 9373-9378, and Sundaramoorthi R, etal, Biopolymers 2003;71(6):717-29). In contrast, AP23236 is a novel ATP-based Src kinase inhibitor also incorporating bone-targeting moieties. Although AP22408 and AP23236 differ mechanistically by virtue of blocking Src-dependent non-catalytic or catalytic activities in osteoclasts, they both address similar indications, and may be useful in the treatment of osteoporosis and related bone diseases, including osteolytic bone metastases and hypercalcemia.

AP23451 is an Src inhibitor identified by Ariad, targeted specifically to the bone to minimize potential inhibitory effects in other organs. This agent exhibited potent *in vitro* and *in vivo* activities, including low nM inhibition of Src tyrosine kinase, and high selectivity against a panel of >30 protein kinases; dose-dependent inhibition of parathyroid hormone-induced hypercalcemia and bone resorption in mice; and prevention of osteolytic lesions in nude mice inoculated with the human breast carcinoma cell line, MDA-MB-231. These findings indicate that AP23451, a novel bone-targeted Src inhibitor, exhibits potent antiresorptive properties both *in vitro* and *in vivo*, suggesting that it may be efficacious in the treatment of osteolytic bone metastases and hypercalcemia of malignancy (Shakespeare W, etal, AACR03, Abs. 3971, and Sawyer TK, etal, ASCO03, Abs. 977).

AP23464

AP23464, also under development by Ariad, is a potent and selective ATP-based inhibitor of both Src and Abl tyrosine kinases. Ariad uses its chemistry drug discovery plat-

Exhibit 1
Drugs in Development Targeting the Src Pathway

Developer ❑ Affiliate(s)	Generic Name ❑ Number ❑ Brand Name	Description ❑ Administration Route	Development Status ❑ Indication(s)
Ariad Pharmaceuticals	AP23464 and analogs	Blocks variant Abl proteins and/or the Src protein ❑ PO	Preclin (ongoing 6/05)➢USA ❑ relapsed or refractory chronic lymphocytic leukemia (CLL)
AstraZeneca	AZD0530	Dual-specific Src/Abl kinase inhibitor with anti-invasion activity in a wide range of tumors ❑ PO	Phase I (ongoing 4/05)➢Europe ❑ advanced solid tumors
AstraZeneca	AZM475271, M475271	Novel, orally available Src kinase inhibitor ❑ PO	Preclin (ongoing 4/05)➢Europe (France, Germany, UK) ❑ solid tumors
Bristol-Myers Squibb	BMS-354825	Small molecule dual function Src/Abl tyrosine kinase inhibitor designed to overcome mechanisms giving rise to resistance to treatment with imatinib mesylate ❑ PO	Phase I (begin 6/03, ongoing 6/05); phase II (begin 12/04, ongoing 6/05)➢USA, Australia, Canada, Europe, China ❑ advanced, refractory CML; phase I (begin 12/04, ongoing 5/05)➢USA, Europe (UK) ❑ refractory gastrointestinal-stromal tumors (GIST) and solid tumors
Centelion	GN963	Small molecule tyrosine kinase inhibitor with activity against PDGFr, Abl, c-Kit, Flt3, and Src kinases ❑ PO	Preclin (ongoing 4/05)➢USA, Europe (France) ❑ solid tumors
Wyeth	SKI-606	Dual specific Src and Abl kinase inhibitor ❑ PO	Phase I (begin 04, ongoing 5/05) ➢USA ❑ advanced solid tumors

Source: NEW MEDICINE's Oncology KnowledgeBASE (nm|OK), June 15, 2005

form, known as SMART (small-molecule Ariad therapeutics) to design its Src inhibitors (Sawyer TK, etal, BioTechniques, Jun 2003;Suppl:2-10, 12-5). The platform combines medicinal chemistry, structure-based drug design and informatics, and biologic target assays. Ariad has developed a cellular assay for inhibitors of protein-protein interactions involving SH2 domains, based on a mammalian cell two-hybrid system. Ariad's mechanism-based cellular assay monitors specific SH2-domain-dependent protein-protein interactions. The assay is a two-hybrid system adapted to function in mammalian cells, where the SH2 domain ligand is phosphorylated, and binding to a specific SH2 domain can be induced and easily monitored. Ariad scientists generated a series of mammalian cell lines that can be used to monitor SH2-domain-dependent activity of the signaling proteins ZAP-70 and Src. These cell lines were then used to screen for immunosuppressive and antiosteoclastic compounds, and to identify small molecule, cell-permeant SH2 domain inhibitors (Rickles RJ, etal, Chem Biol, Oct 1998;5(10):529-538).

AP23464 is being evaluated in the treatment of metastatic malignancies, such as colon cancer, as well as refractory CML. AP23464 demonstrates picomolar inhibition of both Src and Abl kinase activity, and high selectivity (>100,000-fold) against a panel of both tyrosine and serine/threonine protein kinases, and also inhibits multiple human tumor cell lines *in vitro* (Dalgarno DC, etal, AACR-NCI-EORTC03, Abs. A5). AP23464 is over 10-fold more potent than imatinib in blocking the activity of the key cancer-causing protein responsible for imatinib's efficacy. Furthermore, unlike imatinib, AP23464 is designed to block additional therapeutic targets in leukemia that become increasingly important in patients who no longer respond to imatinib.

AP23464 blocked the growth of leukemia cells that harbor any of 30 different mutations in the Abl protein that confer resistance to imatinib. Based on research conducted by George Q. Daley of Harvard Medical School (Boston, MA), AP23464 effectively suppressed even those leukemia cells containing robust and common imatinib-resistant mutants, providing further support for clinical development of AP23464 in patients failing imatinib therapy. Greater potency and broader spectrum of effectiveness of AP23464 make it particularly promising as a treatment of CML, both resistant disease, and as first line therapy.

AP23464 displays antiproliferative activity against a human CML cell line and Bcr-Abl-transduced Ba/F3 cells. It ablates Bcr-Abl tyrosine phosphorylation, blocks cell-cycle progression, and promotes apoptosis of Bcr-Abl-expressing cells. Biochemical assays with purified glutathione S trans-

ferase (GST)-Abl kinase domain confirmed that AP23464 directly inhibits Abl activity. Importantly, the low nanomolar cellular and biochemical inhibitory properties of AP23464 extend to frequently observed imatinib-resistant Bcr-Abl mutants, including nucleotide binding P-loop mutants Q252H, Y253F, E255K, C-terminal loop mutant M351T, and activation loop mutant H396P. AP23464 was ineffective against mutant T315I, an imatinib contact residue. The potency of AP23464 against imatinib-refractory Bcr-Abl and its distinct binding mode relative to imatinib warrant further investigation of AP23464 for the treatment of CML (O'Hare T, etal, Blood, 15 Oct 2004;104(8):2532-9).

Greater potency and broader spectrum of effectiveness of AP23464 make it particularly promising as a treatment of both drug-resistant CML, and as first line therapy for this disease. The common P-loop mutants are sensitive to AP23464, and only the L267R (type Ib) remains resistant. Interestingly, the most frequent and robust mutant T334I (type Ib) was effectively suppressed, whereas F336L (type Ib) is 3-fold resistant over native Bcr-Abl. Surprisingly, 8 of the 30 kinase domain mutants (D295V, E300K, V308S, E311Q, Q319H, T334S, G340W, and M370T-type Ib) were highly sensitive, as compared to the native Bcr-Abl. According to this data, AP23464 appears to be an effective compound against most of the frequently observed mutations of Bcr-Abl in connection with imatinib resistance. Moreover, AP23464 in combination with other drugs may also be a promising therapeutic strategy to overcome imatinib resistance (Mohammad A, etal AACR04, Abs. 4641).

In February 2004, Ariad Pharmaceuticals reported for the first time at the Keystone Conference on Protein Kinases and Cancer, results of studies supporting expansion of the potential clinical indications AP23464, to include specific difficult-to-treat solid tumors, such as breast, ovarian, prostate, liver, lung, kidney, brain, stromal, and colorectal cancer. These studies demonstrate that AP23464 potently blocks the proliferation of cancer cells whose activity is controlled by the key molecular targets of well known marketed oncology drugs, and/or those in late stage clinical development. When tested against over 120 potential molecular targets, AP23464 selectively and potently blocked a limited number of oncogenic proteins, in addition to Src and Abl, which share similar sites of molecular interaction in their drug-binding pocket, including EGFr, HEr2, Raf, kit, PDGFr, and fibroblast growth factor receptor 3 (FGFr3). Previously, AP23464 was being developed solely to treat certain forms of leukemia, based on its inhibition of the Abl protein kinase and its mutant forms, and cancer metastases, based on its inhibition of the Src protein kinase.

AZD0530

AZD0530, a novel anilino quinazoline, is an oral, potent, dual selective inhibitor of Src and Abl in early clinical development as an antimetastatic agent. Structure-activity analysis that led to the discovery of AZD0530 centers on the critical role played by the aniline substitution pattern, and the effect of the C5-C7 quinazoline substitution pattern. AZD0530 possesses excellent properties for a small molecule inhibitor of Src kinase that translate into excellent ADME properties in preclinical animal models. AZD0530 is orally available, and is suitable for once daily administration to humans (Hennequin LF, etal, AACR05, Abs. 2537).

AZD0530 is highly selective for Src and Abl. It exerts its activity through ATP competitive and reversible inhibition of the target enzyme. In addition, AZD0530 is a potent inhibitor of osteoclast-mediated bone resorption, so that in addition to its role as an anti-invasive treatment, AZD0530 may have therapeutic benefit in treating osteoclast-driven metastatic bone disease and osteoporosis (Mullender MG, etal, AACR05, Abs. 2923).

A series of studies were performed to characterize the distribution, PK, and tumor growth inhibitory effects of AZD0530 in rats bearing Src 3T3 xenografts comprising 3T3 mouse fibroblast transfected with a constitutively active human c-Src kinase in an overexpressing vector. Src 3T3 xenograft growth is driven by Src. Dose-related inhibition of tumor growth was seen at various oral dose levels of AZD0530. Drug-related material was extensively distributed to many tissues with high and protracted levels in the xenograft. Following a single oral dose, maximum drug levels of AZD0530 in tumors were approximately 40-fold higher than those in plasma. The shapes of the plasma and tumor AZD0530 concentration versus time curves were similar though the tumor curve lagged behind that of plasma (Logie A, etal, AACR05, Abs. 5989).

AZD0530 causes dramatic inhibition of human breast cancer cell (MDA-MB231) migration *in vitro*. Following a single daily oral administration, AZD0530 completely prevented growth of c-Src-NIH 3T3 xenografts grown in nude rats, and significantly increased survival in an orthotopic model of human pancreatic cancer (BxPC-3).

In a multiple ascending dose trial, conducted in 60 healthy male volunteers to assess the safety, tolerability, and PK profile of AZD0530, enrollees were randomized in cohorts of 12 and treated with AZD0530 (n=9) or placebo (n=3) as a single dose for PK, followed by a washout period, and then by multiple daily dosing for up to 14 days. Doses up to 250 mg/day were administered in this manner. Adverse events were generally mild, including rash, flu-like symptoms, myalgia, arthalgia, headache, loose stools, and raised creatinine. Plasma concentrations remained above the predicted IC_{50} for Src kinases, as predicted from preclinical modeling. AZD0530 was tolerated when administered in multiple daily doses up to 250 mg. The PK of AZD0530 is consistent with once daily oral dosing (Gallagher NJ, etal, AACR05, Abs. 3972).

As of April 2005, an open label, dose-escalation, phase I clinical trial was ongoing at VU Medical Center (Amsterdam, the Netherlands), under PI K. Hoekman, MD, to assess the safety and tolerability of AZD0530 in patients with advanced solid tumors with activated c-Src kinase.

AZM475271 (M475271)

AZM475271 (M475271), under development by AstraZeneca, is an orally active, potent, selective inhibitor of Src kinase. In preclinical testing, conducted at the University of Florida (Gainesville, FL), oral M475271 treatment significantly inhibited the metastatic potential of KHT sarcoma cells in a lung metastasis model. While M475271 treatment led to a significant reduction in the number of metastasis formed, the size of the metastases observed were not significantly reduced. These findings suggest that the primary effect of M475271 may be to inhibit establishment of metastases, rather than to reduce the growth rate of metastatic foci. This conclusion is supported by studies indicating that M475271 treatment has little effect on the growth of macroscopic KHT sarcoma grown intramuscularly (IM). These results indicate that targeting Src kinase activity with agents such as M475271 may provide a promising therapeutic strategy for the treatment of metastatic disease (Shi W, etal, AACR05, Abs. 278).

The effect of AZM475271 was also examined on the growth and metastasis of tumors derived from wild type NBT-II bladder cells. AZM475271 was administered orally to nude mice for 2 months at 2 concentrations that neither resulted in any toxicity. Although AZM475271 interfered only slightly with the growth of tumors derived from injected wild type NBT-II cells, it significantly inhibited the formation of metastases in mice bearing tumors derived from wild type NBT-II cells; only 28% of treated mice harbored lymph node metastases at 2 months after transplantation, compared with 75% in the untreated group. Therefore, AZM475271 prevents wild-type NBT-II bladder cells from metastasizing (Boyer B and Green T, AACR-NCI-EORTC03, Abs. B190).

Investigators at AstraZeneca and the University of Munich-Großhadern LMU (Munich, Germany) observed a synergistic effect of AZM475271 and gemcitabine in human pancreatic cancer growing orthotopically in nude mice. At higher concentrations, AZM475271 significantly inhibited migration of L3.6pl pancreatic tumor cells and migration and sprouting of endothelial cells, and reduced proliferation of human umbilical vein endothelial cells (HUVEC). AZM475271 also has a synergistic effect with gemcitabine in human L3.6pl pancreatic cancer growing orthotopically in nude mice.

Mice were treated with AZM475271 by oral feeding alone, or in combination with intraperitoneal (IP) injection of gemcitabine. Although pancreatic tumor weight was reduced by monotherapy with AZM475271 or gemcitabine compared with untreated controls, the largest reductions were observed in the groups treated with a combination of the two agents, indicating synergism between these drugs. Also, in contrast to untreated controls, no liver metastases were detected following therapy with AZM475271 alone, or in combination with gemcitabine. AZM475271 and gemcitabine exhibited similar synergistic activity in L3.6pl cells *in vitro*. Although the percentage of apoptotic tumor cells was increased after treatment with AZM475271, or gemcitabine, compared with untreated controls, it was considerably greater after treatment with AZM475271 plus gemcitabine compared with either treatment alone. Also, the number of proliferating cells was reduced in tumors treated with AZM475271 alone, or with AZM475271 plus gemcitabine, compared with untreated controls. AZM475271, alone or in combination with gemcitabine, also completely inhibited development of liver metastases *in vivo*, suggesting that Src inhibitors may act as anti-invasive agents in the clinical setting. Microvessel density and proliferation were significantly reduced by AZM475271, but not by gemcitabine. Src kinase inhibitors, such as AZM475271, in addition to inhibitory effects on tumor cells, potentially suppress tumor growth and metastasis *in vitro* and *in vivo* also by antiangiogenic mechanisms (Bruns CJ, etal, AACR-NCI-EORTC03, Abs. B198, and Bruns C, etal, AACR05, Abs. 2021).

Although AZM475271 significantly inhibited migration of human L3.6pl *in vitro*, significantly higher doses were needed to inhibit tumor cell proliferation. However, in contrast to the observations *in vitro*, Src inhibition had antiproliferative activity *in vivo*. In an orthotopic setting, administration of oral AZM475271 in nude mice injected with L3.6pl tumor cells into the pancreas, resulted in a small, but significant effect (~40% inhibition of primary tumor growth) compared to control. AZM475271 inhibited growth of the primary tumor and, in addition, no liver metastases were apparent in AZM475271-treated animals, although liver metastases were present in 60% of mice treated with vehicle alone. AZM475271 did not have a significant effect on tumor vasculature density, indicating that antitumor effects were unlikely to be attributable to inhibition of tumor angiogenesis. Results are consistent with an involvement of Src kinase signaling pathways in tumor cell adhesion and migration phenotypic changes. They also support the concept that Src kinase inhibitors may have potential as anti-invasive agents. Determining whether this is a direct or indirect consequence of Src kinase inhibition in the primary tumor will require further investigation (Yezhelyev M, etal, AACR03, Abs. R1718).

The apparent discrepancy in explaining the mechanism of action of the same Src inhibitor in a similar *in vivo* model, underscores the challenge involved in understanding how inhibition of various points in complex pathways, in somewhat differing circumstances, affects tumor growth and metastasis.

BMS-354825

BMS-354825, a 2-substituted aminopyrimidinyl-thiazole-5-carboxamide analog, is an ATP-competitive, small molecule tyrosine kinase inhibitor that suppresses SFK, as well as EphA2, Abl, c-kit, and PDGFr activity at subnanomolar concentrations. First and foremost, BMS-354825 has been designed as an alternative and/or extension to imatinib-addressed indications. BMS-354825 effec-

tively inhibited proliferation of cells expressing nearly all imatinib-resistant isoforms, suggesting that this compound has a different set of structural requirements for Abl kinase inhibition than imatinib (Shah NP, etal, AACR04, Abs. 5624). BMS-354825 is considered the next generation of agents like imatinib. It has been refined and improved using structural biology so that the drug 'fits' its target, and is also effective against mutations that may alter the shape of the target.

Unlike imatinib, BMS-354825 binds to the active form of Abl, which closely resembles the active configuration of Src. Imatinib does not bind to the active form of the Bcr-Abl fusion, thereby allowing resistance to arise when mutations lock Abl in an active state. BMS-354825 exhibits 100-fold greater potency in inhibiting Bcr-Abl *in vitro* than imatinib, and demonstrated *in vitro* and *in vivo* activity against 14 of 15 imatinib-resistant Bcr-Abl mutants (Shah NP, etal, Science, 16 July 2004;305 (5682);399-401).

The central cores of BMS-354825 and imatinib occupy overlapping regions, but these two inhibitors extend in opposite directions. The activation loop remains in the active conformation in the presence of bound BMS-354825 in contrast to bound imatinib. There are also no steric clashes that would preclude BMS-354825 from also binding to the inactive conformation of the activation loop, suggesting that the increased binding affinity of BMS-354825 over imatinib is at least partially attributable to its ability to recognize multiple enzyme states. The P-loop is partially disordered, suggesting that interactions between this part of the protein and BMS-354825 are less critical for binding. Interestingly, several imatinib-resistant mutations occur in the P-loop (Tokarski JS, etal, ASH04, Abs. 553).

BMS-354825 is 300 to 650 times more potent than imatinib in the K562, KU-812, MEG-01, and SUP-B15 Bcr-Abl-dependent leukemic cell lines. Moreover, BMS-354825 is equally effective against several preclinically and clinically derived tumor models of imatinib resistance. BMS-354825 induces apoptosis in both the imatinib sensitive K562 (Bcr-Abl+, Lyn-) and resistant K562R (Bcr-Abl+, Lyn+) cell lines, correlating with inhibition of both Lyn activation and Bcr-Abl signaling. BMS-354825 effectively reduces both K562 and K562R tumor growth *in vivo*, whereas imatinib has minimal effects on K562R tumors. BMS-354825 completely retains its effectiveness against the K562R model, which has a 6-fold resistance to imatinib, and also overexpresses Fyn, a SKF member.

While imatinib has minimal inhibitory effects on Lyn/Hck activation in clinical specimens taken from patients with imatinib-resistant CML, BMS-354825 completely suppresses Lyn/Hck phosphorylation, which correlates with its greater antitumor activity. BMS-354825 reduces both Lyn and Bcr-Abl activation in co-expressing cells, suggesting that Lyn-mediated phosphorylation plays a direct role in imatinib resistance (Donato NJ, etal, ASH04, Abs. 1989). Recently established CML cell lines from patients with imatinib-resistant CML also demonstrated greater apoptotic sensitivity when exposed to BMS-354825 *in vitro*, which correlated with inhibition of Bcr-Abl and overexpressed and activated Src-related kinases in CML cells (Wu JY, etal, AACR04, Abs. 3850).

Simultaneous inhibition of Src and Bcr-Abl kinases and their downstream pathways may also be effective in treating patients with Philadelphia chromosome-positive (Ph+) B-cell acute lympholytic leukemia (B-ALL). Although Src inhibitors have some effect on B-ALL, they alone may not cure the disease. However, BMS-354825 that targets both Src and Bcr-Abl kinases completely eradicated leukemia cells in mice with B-ALL (Li S, etal, ASH04, Abs. 1976).

Also, unlike imatinib, BMS-354825 is not susceptible to the p-glycoprotein (P-gp) efflux pumping mechanism that has been linked to imatinib resistance preclinically (Lee FY, etal, AACR04, Abs. 3987). Therefore, BMS-354825 crosses the blood brain barrier (BBB) as evidenced in an *in vivo* model of established intracranial CML tumors. The drug increases the life span of these mice by 450% and 268% for the 15 mg/kg and 5 mg/kg dose levels, respectively. BMS-354825 also achieves tumor regressions and subsequent complete stasis of intracranial K562 growth while animals remained on therapy (Wild R, etal, ASH04, Abs. 1988).

BMS-354825 is also effective in various solid tumors. BMS-354825 competes with ATP for the ATP-binding site in the kinase domain of selected and related oncogenic receptor and nonreceptor protein tyrosine kinase (PTK), including Bcr-Abl, SFK (Src, Lck, Yes, Fyn), c-Kit, EphA2, and PDGFβr, which are dysregulated in many solid tumors, resulting in uncontrolled proliferation, inappropriate adhesion, heightened motility and metastatic potential, resistance to apoptosis, and angiogenesis promotion. In *in vitro* kinase assays, BMS-354825 potently inhibits these PTK at subnanomolar to low nanomolar concentrations. In cellular assays, BMS-354825, at clinically achievable concentrations, inhibited proliferation of 11/23 (48%) lung, 9/31 (29%) colon, and 7/23 (30%) breast cancer cell lines. In preclinical chemotherapy studies against a random panel of 13 solid tumors grown in mice, BMS-354825 significantly inhibited the growth of 6/13 (46%) xenografts at doses producing drug exposure similar to those currently achieved in ongoing phase I clinical trials. Responsive tumor types included breast, prostate, colon, and pancreatic cancer, small cell lung cancer (sclc), and sarcoma (Lee FY, etal, AACR05, Abs. 675).

BMS-354825 may also be effective in head and neck cancer and nsclc. Incubation of head and neck squamous cell carcinoma and nsclc with clinically relevant BMS-354825 concentrations resulted in multiple biological effects that appear to be mediated through the inhibition of Src and/or EphA2. BMS-354825 inhibited migration in all cell lines, and induced cell-cycle arrest by blocking the G1 to S transition leading to apoptosis in highly sensitive cell lines. Effects on migration correlate with inhibition of Src and downstream mediators of adhesion such as Fak, p130, and paxillin, while cell-cycle effects and apoptosis correlate

with inhibition of Akt, induction of p27, and dephosphorylation of Rb. BMS-354825 also induced morphologic changes that were integrin-dependent, consistent with an upstream role for Src in regulation of focal adhe sion complexes (Johnson F, etal, AACR05, Abs. 674).

David S. Hong, MD, at M. D. Anderson Cancer Center, has shown that BMS-354825 also inhibits Src kinase in pancreatic cancer cells *in vitro*. Inhibition of Src led to inhibition of vascular VEGF in these cells. This hypothesis is being tested further *in vitro* and *in vivo* to see if BMS-354825 kills isolated pancreatic cancer cells alone, or in combination with gemcitabine.

Clinical trials with BMS-354825 are ongoing in hematologic malignancies and solid tumors. Promising results from phase I clinical trials supporting the safety and efficacy of BMS-354825 in advanced, imatinib-resistant CML, have spurred the initiation of a comprehensive worldwide clinical program, investigating the drug in phase II clinical trials as monotherapy, and in randomized phase II clinical trials against imatinib, in chronic, accelerated, and blast crisis CML. Response to imatinib is particularly inferior in chronic phase (CML-CP), accelerated phase (CML-AP), and blast phase (CML-BP) CML.

In phase I clinical trials, BMS-354825 was administered to patients with Ph+ CML (CML-CP=36, CML-AP=8, and CML-BP=21) resistant or intolerant to imatinib. Among those with chronic phase CML, the complete hematologic response (CHR) was 86%. Among 29 patients for whom cytogenetic data were available, there were 8 (28%) major cytogenetic responses. In the CML-AP group, hematologic response was 75%, but there were no cytogenetic responses. In the CML-BP group, hematologic response was 79%. In this group, among 15 patients for whom cytogenetic data were available, a major cytogenetic benefit was observed in 53%.

Regarding toxicities, among those with chronic phase CML, Grade 4 thrombocytopenia requiring treatment modification was seen in 3/26 patients, and GI bleeding, possibly related to BMS-354825, was reported in 2. In the CML-BP group, evidence of tumor lysis syndrome was reported in 2 patients, and 1 patient with CML-AP contracted pneumonia, possibly related to BMS-354825. In all phases, additional side effects reported during the trials included arthralgia, pyrexia, fatigue, peripheral edema, headache, and diarrhea, and mild prolongation of the QT interval. At the time of this report, no patients had discontinued treatment in the phase I trial because of toxicity.

One concern regarding inhibition of Src is toxicity related to the fact that SFK are important modulators of T-cell activation. Therefore, effects of BMS-354825 on T-cell cytokine synthesis was evaluated in patients with imatinib-resistant chronic phase CML treated with BMS-354825. Blood tests were performed on 14 patients with chronic phase CML on days 1 (baseline), 5, and 26 of course 1 of treatment with BMS-354825, at total daily doses ranging from 15 mg to 180 mg, administered as a single or divided daily dose, to evaluate intracellular cytokine synthesis of Th1 (IL-2, IFN-α, TNF-α) and Th2 (IL-10) cytokines. In 13/14 patients with CHR, no changes were detected in distribution of T-cell subsets, regardless of the dose and dosing schedule. Therefore, BMS-354825 treatment in this setting effectively induced CHR without affecting the ability of CD4+ T and CD8+ T cells to synthesize Th1 and Th2 cytokines *in vitro* (Gao H, etal, ASCO05, Abs. 6619).

In an open label, dose-escalation, phase I clinical trial (protocol IDs: CDR0000310142; UCLA-0303035; BMS-CA180002), initiated in June 2003, BMS-354825 is being administered to patients with Ph+ CML-CP resistant or intolerant to imatinib. This trial was initially restricted to CML-CP, but was subsequently amended to include patients with CML-AP, CML-BP, and Ph+ ALL. Intrapatient dose escalation is also permitted. According to the protocol, BMS-354825 is administered PO once daily for 5 days, repeated every 7 days, for at least 3 months, in the absence of disease progression or unacceptable toxicity. Cohorts of 3 to 6 patients are being administered escalating doses of BMS-354825 until MTD is determined. An additional 20 patients are then to be treated at MTD. The trial is to accrue 50 patients, 30 in phase I, and 20 in phase II. Charles Sawyers, MD, of the Jonsson Comprehensive Cancer Center at UCLA (Los Angeles, CA) is the trial's PI. The trial is seeking to determine PK, Bcr-Abl mutations, and CrkL, Hck, and Lyn phosphorylation.

From May through November 2004, 29 patients (CML-AP=8, CML-BP=18, and Ph+ ALL=3) were treated twice daily with 35 mg to 90 mg doses of BMS-354825. Imatinib-resistance mutations were identified in 16 of 28 patients with mutation data. Complete and partial hematologic response rates were 75% (6/8) for CML-AP, 76% (13/17) for CML-BP, and 100% (2/2) for Ph+ ALL. Responses were durable for 2+ to 6+ months in 19 patients. The major cytogenetic response rate in patients with CML-BP was 53%. A total of 15 patients with imatinib-resistance mutations responded. Among these, 4 patients had primary resistance documented after 2 months, and 2 relapsed within 2-3 months after an initial response. The T315I mutation, which confers cross-resistance to both drugs in preclinical models, was identified in 4 of the 6 patients with resistant disease. In terms of toxicities, BMS-354825 was well tolerated. Myelosuppression, with Grade 4 thrombocytopenia, was seen in 8 patients. Grade 3/4 fluid retention (n=1) and tumor lysis syndrome (n=2) occurred in 3 patients (Sawyers CL, etal, ASCO05, Abs. 6520).

Among 36 patients with CML-CP (resistant=31, intolerant=5), treated with total daily doses of BMS-354825 (range=15-180 mg), administered as single or divided dose, mutations associated with imatinib resistance were identified in 27 patients. A complete CHR occurred in 31 (86%) patients, with disease progressing in only 2. A cytogenetic improvement was reported in 13/29 (45%) patients, including 9 major cytogenetic responses (MCyR), 5 complete and 4 partial, for a MCyR rate of 31%. Hematologic and cytogenetic responses occurred regardless of mutation status, or

whether the mutation was in the P-loop, catalytic, or activation region, with the exception of 1 patient with a T315I mutation whose disease progressed. Responses are durable, with 33/36 patients remaining on the trial for 1+ to 13+ months. Regarding toxicities, there were 3 cases of Grade 4 thrombocytopenia, and 2 of Grade 4 neutropenia, all reversible and easily managed with dose modification. A duodenal ulcer developed in 1 patient, possibly related to BMS-354825. Mild QTc prolongation has also been noted (Talpaz M, etal, ASCO05, Abs. 6519).

As of August 2004, 17 patients (CML-AP=6 and CML-BC=11) were treated at M. D. Anderson Cancer Center, under PI Moshe Talpaz, MD, with BMS-354825 in 3 cohorts, at doses ranging from 35 mg to 70 mg twice daily. BMS-354825 was rapidly absorbed, with peak concentrations achieved within 2 hours, and a terminal phase half-life of about 5 hours. A consistent, rapid, and sustained inhibition of Lyn kinase was observed. To date, BMS-354825 has been very well tolerated.

Among 11 patients with CML-BP, there were 7 hematologic responses, 3 CHR, 2 'no evidence of leukemia' (NEL), and 2 'return to chronic (RTC) phase'. A significant hematologic improvement was seen in 3 additional patients, despite being on treatment for only a short period of time (range=10-23 days). Disease stabilized in 1 patient with extramedullary disease. Among 8/11 patients with CML-BP for whom cytogenetic data is available, there were 4 major cytogenetic responses, and 2 minor cytogenetic responses; no responses were noted in 2 patients. Among 2 patients evaluated for Bcr-Abl mutations, 1 did not have a mutation, and the other who had a nonsustained CHR, had an E355G mutation.

Among patients with CML-AP, a hematologic response was seen in 3/6, 2 CHR, and 1 NEL; 2 patients were too early to assess, and disease was resistant to BMS-354825 in 1 patient because of a T315I mutation in Bcr-Abl in 8 of 10 clones, which conferred resistance to BMS-354825 in preclinical studies. Among 3 other patients with CML-AP for whom Bcr-Abl mutation status is available, no mutations were present in 2, and 1 in CHR had M351T/A imatinib-resistant mutations. Of 3 patients with CML-AP for whom early cytogenetic data is available, there was 1 minor cytogenetic response (40% Ph+). Additional clinical trials are required to establish Lyn's potential role in imatinib-resistant CML (Talpaz.M, etal, ASH04, Abs. 20).

Clinical activity of BMS-354825 in imatinib-resistant CML and Ph+ ALL was observed in patients with a wide range of imatinib-resistant Bcr-Abl kinase domain mutations. In the phase I clinical trial, prior to therapy, peripheral blood samples were collected for mutation analysis for correlation with response. Among 63 evaluable patients (CML-CP=36, CML-AP=8, CML-BP/Ph+ ALL=19), 17 different imatinib-resistant point mutations in the Bcr-Abl kinase domain were identified in 67% of patients prior to therapy. CHR was observed in patients harboring each of these mutations except T315I (n=5), F317L (n=1), and D276G (n=1). A PR was observed in 2 patients with F317L and D276G mutations who remained on trial. The T315I mutation is highly resistant to BMS-354825 in experimental systems. Among 9 patients with progressive disease (PD) 3 harbored T315I mutations detected prior to treatment, and, in 2, the T315I mutation was detected at the time of disease progression. Assessment of Bcr-Abl kinase inhibition in patient samples was correlated with response, and loss of Bcr-Abl kinase inhibition was correlated with relapse. The T315I mutation, which is cross-resistant to both BMS-354825 and imatinib, as predicted from preclinical studies, appears to be the only Bcr-Abl kinase domain mutation resistant to BMS-354825 (Shah N, etal, ASCO05, Abs. 6521).

BMS-354825 is also being investigated in solid tumors. A dose-escalation, phase I clinical trial (protocol ID: CA180-003) of BMS-354825 in patients with GIST and other solid tumors, was initiated in December 2004, at the Dana-Farber Cancer Institute (Boston, MA) and Beatson Cancer Center (Glasgow, Scotland). This is the first trial to evaluate the safety, tolerability, and pharmacologic profiles of BMS-354825 in patients with treatment-resistant GIST and other refractory solid tumors. Patients with adequate hematologic, renal, cardiac, and liver function, are being treated with BMS-354825 orally twice daily for 5 days, followed by a 2-day rest, every week. Patients in alternate dose groups are treated after a fast or high fat meal plus antacids. Patients are assessed continuously for safety; EKG monitoring is performed on days 1, 5, 26. PK profiling is performed on days 1, 8, 26, and PD biomarkers (pSrc, pFak, Kit, and Scf) and early imaging with FDG-PET are performed in the first week of treatment, with a PD biomarker assessment on day 26 as well. Serial imaging with CT is performed at least every 8 weeks, with FDG-PET in weeks 1, 4, and 8.

Among 14 patients with GIST (n=9) or other solid tumors (n=5), treated in 1 of 3 escalating dose levels (35, 50, or 70 mg) twice daily, toxicity included clinically insignificant Grade 3 lymphopenia (n=2), Grade 3 anorexia (n=1), and Grade 3 elevation of alkaline phosphatase (n=1). There was no DLT. At the 70 mg twice daily dose, the PK profile in fasting patients resembles that of fasting patients with hematologic malignancies, but food and GI pH show an effect on PK parameters. There were no objective responses on CT, but activity has been noted as mixed responses on FDG-PET, and resolution of GIST-associated ascites (n=1); 2 patients with GIST remain on treatment for >3 months. BMS-354825 can be safely administered at doses of up to 70 mg, twice daily on this schedule. Dose escalation continues (Evans TR, etal, ASCO05, Abs. 3034).

In addition, an open label, multicenter, randomized, phase II clinical trial (protocol IDs: CDR0000428457; UCLA-0501047-01; BMS-CA180017; EUDRACT-2004-004450-96; NCT00112775) with BMS-354825 was initiated in 2004, at the Jonsson Comprehensive Cancer Center at UCLA, under PI Neil P. Shah, MD, to treat patients with chronic phase CML that did not respond to previous treat-

ment with imatinib. According to the protocol, patients are stratified according to trial site and cytogenetic response to prior imatinib treatment (yes versus no). Patients are randomized to 1 of 2 treatment arms. In arm I, patients are treated with oral BMS-354825 twice daily in the absence of disease progression or unacceptable toxicity. Those experiencing disease progression or persistent intolerance to BMS-354825 cross over to arm II after a 2-day washout period. After crossover, patients are treated with oral imatinib twice daily in the absence of further disease progression or unacceptable toxicity. In am II, patients are treated with oral imatinib twice daily in the absence of disease progression or unacceptable toxicity. Those experiencing disease progression, intolerance to imatinib, lack of major cytogenetic response at 12 weeks, or <30% absolute reduction in Ph+ metaphases at 12 weeks, cross over to arm I after a 1-week washout period. After crossover, patients are treated with oral BMS-354825 twice daily in the absence of further disease progression or unacceptable toxicity. Quality of life (QoL) is assessed at baseline, at day 29, every 4 weeks for 24 weeks, every 12 weeks for the remainder of the trial, at the completion of the trial, and for at least 30 days thereafter.

This trial's primary objective is to determine the 12-week MCyR rate in patients with imatinib-resistant CML-CP treated with BMS-354825 versus imatinib. Secondary objectives are to determine the MCyR rate and durability and time to MCyR prior to crossover, as well as the CHR rate, the durability of CHR, and time to CHR prior to crossover. Other objectives are to determine the major molecular response rate prior to crossover, as determined by Bcr-Abl transcripts in blood during treatment, and the post-crossover efficacy endpoints in patients treated with these drugs who crossover. The trial will also assess QoL and determine PK and safety and tolerability of BMS-354825 in these patients. A minimum of 150 patients (arm I=100 and arm II=50) will be accrued for this trial.

GN963

GN963, under development by Centelion (Vitry sur Seine, France), an affiliate of sanofi-aventis, is a small molecule tyrosine kinase inhibitor with activity against PDGFr, Abl, c-Kit, Flt3, and Src kinases.

Investigators at M. D. Anderson Cancer Center determined that the optimal dose and schedule of GN963 administered orally to inhibit phosphorylation of the PDGFr in tumor cells and tumor-associated endothelial cells, was 100 mg/kg, administered every 48 hours. In nude mice injected with L3.6pl cells, treatment with gemcitabine did not significantly differ from control, while treatment with GN963 (100 mg/kg) produced a 63% decrease in pancreatic tumor weight, and treatment with GN963 and gemcitabine produced a 73% decrease in tumor weight, and reduced the incidence of liver metastasis from 8/10 to 2/10. This treatment inhibited phosphorylation of the PDGFr, Akt, and Src on both tumor cells and tumor-associated endothelial cells, and decreased microvessel density, decreased proliferating cell nuclear antigen staining, while increasing apoptosis of tumor cells and tumor-associated endothelial cells (Baker CH, etal, AACR05, Abs. 258).

SKI-606

SKI-606 is a small molecule dual specific Src and Abl kinase inhibitor, under development by Wyeth. SKI-606 belongs to a new class of synthetic compounds, 4-anilino-3-quinolinecarbonitriles. SKI-606 is based on variations of the substituents on the 4-anilino group of the 3-quinolinecarbonitrile core. Wyeth discovered that 4-(3-bromoanilino)-6,7-dimethoxy-3-quinolinecarbonitrile was a potent and selective inhibitor of EGFr, now in development as EKB-569, an irreversible EGFr inhibitor. The variation changed the kinase specificity from EGFr to either Src or MEK. SKI-606, initially thought to only inhibit Src, was subsequently found to also inhibit Abl kinase and, therefore, could be a potential alternative treatment to imatinib.

SKI-606 inhibits anchorage independent and invasive growth in tumor cells. It reduces tumor size in nude mice. SKI-606 inhibits phosphorylation of cellular proteins, including STAT5, at concentrations that inhibit proliferation in CML cells.

SKI-606 ablates tyrosine phosphorylation of Bcr-Abl in CML cells and of v-Abl expressed in fibroblasts. Phosphorylation of the autoactivation site of the SFK Lyn and/or Hck is also reduced by treatment with SKI-606. Once daily oral administration of this compound causes complete regression of large K562 xenografts in nude mice (Golas JM, etal, Cancer Res, 15 Jan 2003;63(2):375-81).

Investigators at the University of Bologna, in Italy, analyzed human cell lines from patients with CML-BP (K562, MK2, LAMA84) and CD34+ from 8 patients in CML-BP, using a wide range of concentrations of SKI-606. A decrease in cell viability was observed in K562, MK2, and LAMA84 after treatment with SKI-606. Also apoptotic cell death was associated with an accumulation in the G1/S phase. The antiproliferative and cytostatic effects of SKI-606 were confirmed by a general decrease of Bcr-Abl expression, and reduced protein tyrosine phosphorylation, including hypophosphorylation of Lyn and Hck. An increase of Bax, a decrease of Bcl-xl expression, and a subsequent activation of caspase-9 were also observed (Grafone T, etal, AACR05, Abs. 5984).

A dose-escalation, phase I clinical trial (protocol ID: WAGM 1Y04) of oral SKI-606 was initiated in 2004, at the Ireland Cancer Center (Cleveland, OH) under PI Smitha S. Krishnamurthi, MD, in patients with advanced solid tumors. Trial objectives are to evaluate the safety and tolerability of oral SKI-606 administered on a daily schedule to patients with advanced solid tumors, and to define MDT. According to the protocol, patients take SKI-606 PO in the morning with water and food as a single dose on day 1, and as continuous once daily dosing from day 3. All patients are being treated with SKI-606 until disease pro-

gression, unacceptable toxicity, or withdrawal of consent for up to 9 cycles. Patients with evidence of clinical benefit (SD or better) after 9 cycles may continue to be treated with SKI-606.

Other Programs

Various academic and commercial entities are pursuing programs targeting SKF and related kinases.

Investigators at Kyowa Hakko Kogyo (Tokyo, Japan) developed UCS15A, a novel small molecule that disrupts SH3 domain-mediated protein-protein interactions. UCS15A, produced by Streptomyces species, inhibited proline-rich ligand-mediated protein-protein interaction (PLPI). This ability of UCS15A appears to be restricted to SH3-mediated protein-protein interactions. In this regard, UCS15A is the first example of a nonpeptide, small molecule agent capable of disrupting SH3-mediated protein-protein interactions. *In vitro* analysis suggests that UCS15A does not bind to the SH3 domain itself, but rather interacts directly with the target's proline-rich domains (Oneyama C, etal, Oncogene 27 Mar 2002;21(13):2037-50).

One of the biologic consequences of Src-inhibition by UCS15A is its ability to inhibit the bone resorption activity of osteoclasts *in vitro*. This may be accomplished, not by inhibiting Src activity, but by disrupting the interaction of proteins associated with Src, thereby modulating downstream events in the Src signal transduction pathway (Sharma SV, etal, Oncogene, 19 Apr 2001;20(17):2068-79).

Synthetic analogs of UCS15A were more potently active than natural UCS15A in inhibiting PLPI, and unlike natural UCS15A, the analogs were an order of magnitude less cytotoxic, and did not cause morphologic changes in treated cells (Oneyama C, etal, Chem Biol, May 2003;10(5):443-51).

Investigators at ImClone Systems (New York, NY) constructed and expressed single domain antibodies directed against Etk kinase domain, isolated from a phage display library. Dominant-negative inactivation of Etk results in a reduction in the transforming activity of v-Src in NIH3T3 cells. These domain antibodies interacted with Etk kinase domain, blocking its enzymatic activity. Lipofectamin transfection and introduction of the single domain antibodies into NIH3T3 cells overexpressing v-Src resulted in intracellular expression of the antibodies (intrabodies), decreasing Etk intrinsic kinase activity. These findings indicate that Etk plays a role in Src-induced cellular transformation, and suggest the potential of intracellular antibodies in targeting cytoplasmic signaling molecules (Brennan L, etal, AACR05, Abs. 564).

In January 2003, Signase, now defunct, and Tripos (St. Louis, MO) were granted jointly by the USPTO patent #6,503,914, titled "Thienopyrimidine-based inhibitors of the Src family." This patent seems to have reverted back to the University of Texas (Austin, TX) system.

Editor's note: *Upcoming issues of FUTURE ONCOLOGY will describe ongoing monotherapy phase I clinical trials of novel agents classified by major mechanism of action and target, if applicable, reported during the 2005 meetings of the American Association of Cancer Research (AACR) and the American Society of Clinical Oncology (ASCO).*

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FUTURE ONCOLOGY

PUBLISHED BY **NEW MEDICINE, INC.**

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SUBSCRIPTION INFORMATION:

- FUTURE ONCOLOGY (ISSN 1082-331X) is published as 12 issues (several double issues) per subscription period, with a free index listing companies/institutions and subjects covered.
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- Volumes V3, V4, V5, V6, and V7 are $2,400 (U.S.) and $2,460 (outside the U.S.), either electronic or hard copy versions.
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