The Development of Molecularly Targeted Anticancer Therapies: An Eli Lilly and Company Perspective

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Abstract: The ability to identify activated pathways that drive the growth and progression of cancer and to develop specific and potent inhibitors of key proteins in these pathways promises to dramatically change the treatment of cancer: A patient's cancer could be characterized at the molecular level and the information used to select the best treatment options. The development of successful therapies not only requires extensive target validation, but also new approaches to evaluating drug efficacy in animal models and in the clinic compared to the development of traditional cytotoxic agents. This article highlights Eli Lilly and Company's approach to developing targeted therapies, from target identification and validation through evaluation in the clinic. A selection of drugs in the Lilly Oncology pipeline is also discussed.

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Traditional Cancer Drug Development

The first effective cancer chemotherapies resulted from studying the effects of mustard gas (sulfur mustard) used as a chemical warfare agent in World War I.[1] In 1929, it was reported that skin irritations produced by diluted sulfur mustard solutions did not enhance the appearance of skin tumors in mice topically treated with carcinogenic tar as expected, but instead blocked tumor formation.[2] This and other animal studies led to the first clinical trial of sulfur mustard in 1931 in 13 patients with skin cancer. Sulfur mustards produced some complete responses through topical application or injection into tumors.[3] Investigations of the more stable nitrogen mustards during World War II led in 1942 to the first clinical trial of systemic nitrogen mustard therapy for the treatment of lymphosarcoma and other cancers.[4-6] Nitrogen mustard is still in clinical use today. In the late 1950s, Lilly researchers codiscovered the vinca alkaloid class of anticancer agents in the flowering periwinkle (*Catharanthus roseus* G. Don) through screens of plant extracts for antitumor activity.[7] Lilly introduced 2 of these alkaloids, vincristine sulfate (Oncovin) in 1963 and vinblastine sulfate (Velban) in 1965, for the treatment of acute leukemia.

Much of the early effort in cancer drug development was focused on identifying cytotoxic agents that could potentially eradicate cancer cells in the body through short-term systemic delivery. Over the last 50 years, millions of chemicals and biological extracts have been screened for anticancer activity in vitro against cell lines derived from tumors and hematologic malignancies. Compounds with sufficient activity in vitro were often tested in mice bearing tumors derived from human cancer cell lines (xenograft models). In these assays, investigators looked for evidence of tumor shrinkage with acceptable toxicities relative to the effects of other compounds and vehicle-treated controls. Promising compounds were selected for preclinical and clinical development.

While many of the cancer chemotherapy drugs in clinical use were developed using this approach, many more molecules failed to show enough clinical activity or a sufficient therapeutic index to warrant further development. The high failure rate is partly due to the shortcomings of xenografts as models of human cancer. Cell lines used in xenograft studies are selected for fast growth in vivo and may poorly model the drug sensitivity of slow-growing cancers. In fact,

experiments at the National Cancer Institute showed that only 18 of 48 human cancer cell lines grown as tumors in mice were sensitive to any of 12 chemotherapeutic agents tested, where sensitivity was defined as tumor shrinkage of at least 50%.[8] While new approaches are being used to create tumor models that better recapitulate certain aspects of human cancers,[9] it is too early to tell whether these models will be better than xenografts at predicting clinical efficacy of anticancer drugs. However, the clinical success rate of new drugs is likely to improve as the focus of drug development shifts from the search for traditional cytotoxics to molecularly targeted therapies.

Molecularly Targeted Therapies

An exciting alternative approach to cancer drug development is to identify specific inhibitors of signaling events that play a critical role in cancer cells or the tumor microenvironment. Molecularly targeted agents, unlike many cytotoxic therapies, could be matched to patients who are likely to benefit from treatment through diagnostic screening, a strategy known as "personalized medicine." Many targeted therapies will result in inhibition of cancer cell growth (cytostasis) as opposed to induction of apoptosis and tumor shrinkage. With an appropriate toxicity profile, such therapies could be administered on a prolonged, continuous basis with the goal of turning cancer into a chronic disease with an improved quality of life. These differences require changes in the way the efficacy of targeted therapies are assessed during preclinical and clinical development. We will discuss the key aspects of developing successful targeted agents, beginning with target identification and validation.

Target Identification/Validation

The success of a new, targeted therapy depends upon identifying a key regulator of cancer cell growth, survival, proliferation, or metastasis in the cancer of interest. Distinguishing between proteins that provide a critical function in the tumors of a significant number of patients and proteins with redundant functions or those that are only necessary for tumor initiation is challenging. It is now feasible to use a systems-biology approach to integrate large amounts of data from many different sources to minimize the biases of any particular model, to highlight activated pathways, and to begin to understand how molecular events at the gene and protein levels are connected to global events in the cancer cell and tumor and to the physiological processes of the body.[10,11] This includes data from genomic (DNA), transcriptomic (gene expression at the RNA level), proteomic (protein expression), metabolomic (metabolite profiling), and clinical (associated disease correlates) analyses. Investigations of biological processes that are important to cancer growth and survival, including programmed cell death (apoptosis), cell cycle regulation, growth and proliferation signal transduction pathways, and angiogenesis, have identified many potential sites for therapeutic intervention.

Potential targets should be frequently overexpressed or have increases in activated protein in human cancers of 1 or more tissues so that a large number of patients would potentially benefit from therapy. Evidence of frequent genetic activation of the target through loss of a regulator (by mutation, deletion, or methylation), gene amplification, formation of fusion genes (through deletions or translocations), or mutations that result in activated proteins strongly supports the hypothesis that overexpression or activation of the protein is physiologically important in cancer. The target may also become activated by similar mutations in upstream members of the same signal transduction pathway. Inactivating mutations or deletions of the phosphatase and tensin homolog (PTEN) tumor suppressor gene, for example, results in hyperactivation of phosphatidylinositol 3 kinase (PI-3K), which leads to constitutive activation of the downstream kinases Akt (also known as protein kinase B) and mammalian target of rapamycin (mTOR).[12] Thus, characterization of activated pathways is a good strategy for identifying appropriate targets.

Direct Modulation of the Target

It may be possible to demonstrate that direct modulation of some targets in cell culture or in mouse tumor models has an important effect on the biology of the cancer cell. This kind of evidence comes from experiments where expression of the target gene is experimentally increased or decreased, and where the activity of the target protein is inhibited in cell lines or mouse tumor models. For example, a mouse model could be generated that develops tumors spontaneously during the life of the animal and in which expression of the gene of interest can be induced by adding the antibiotic doxycycline to the animal's drinking water.[9] If induction of this gene decreases tumor latency, or increases tumor growth rate or metastasis, this would support a physiologic role for the gene in cancer. Another approach is to generate mice that carry an activated form of a gene that is only expressed in cells that undergo a spontaneous recombination event. This was used to create a model of spontaneous K-*ras* oncogene activation that is highly predisposed to a range of tumor types, predominantly early onset lung cancer.[13] Alternatively, the overexpression of a gene in a cell line through transfection and selection of stable transfectants is commonly used to demonstrate the effects of overexpressing a single gene on the phenotype of the cells.

Antisense oligonucleotides (ASOs) can be used to inhibit the translation of or elicit the destruction of a gene's messenger RNA (mRNA)—thereby inhibiting production of the gene's protein—in experiments performed in cell culture or in mouse models.[14,15] Recombinase-mediated gene deletions[9] and RNA inhibition (RNAi)–mediated silencing[15,16] of particular genes can also be used to inhibit a target at the level of gene expression. Some targets can be inhibited at the protein level by expression of dominant-negative forms of the protein that effectively interfere with function of the normal protein. Examples include a truncated transforming growth factor-β receptor II mutant that is incapable of phosphorylating substrates[17] and a terminally truncated transcription factor hepatocyte nuclear factor-1 β (HNF1β) that can dimerize with wild-type HNF1β but cannot bind DNA.[18]

These kinds of data are valuable in discovering a target's mechanism of action, exploring its potential physiologic role, and identifying models for preclinical development. However, we do not view such data as a prerequisite to high-throughput screening if the target is sufficiently validated by other means.

Preclinical Evaluation of a Targeted Agent

A typical approach to identifying inhibitors of molecular targets includes a primary biochemical assay with a recombinant protein or engineered mammalian cells followed by cell-based in vitro assays and in vivo assays in a cancer disease model.[19] A key part of developing targeted therapies is the measurement of target inhibition at a point as close to the target as possible. For example, the phosphorylation status of kinase substrates in the presence of an inhibitor could be analyzed by probing Western blots with phospho-specific antibodies. For genes targeted with ASOs, destruction of the mRNA and decreases in the target protein levels can be assayed. Target inhibition assays are a key part of all phases of Lilly Oncology's development of targeted therapies, including in vivo evaluation of compounds as discussed below. The selectivity (activity against the target versus related isoforms or protein family members) and specificity (activity for the target versus unrelated targets in the same protein category) of lead compounds should also be measured. Cell-based efficacy models that measure the effect of a compound on a biologic endpoint such as growth or apoptosis can be used to further validate a target. However, off-target effects of a compound could obscure the true effects of inhibiting the target if used too early in the lead optimization process.

In Vivo Target Inhibition

It is possible that specific inhibition of a drug target in xenograft models will result in tumor shrinkage, tumor growth inhibition, apoptosis, inhibition of angiogenesis or metastasis, or other measurable biologic responses. However, the key objective of xenograft studies at Lilly is to demonstrate in vivo target inhibition. Mice bearing human cell line–derived tumors are treated with the compounds to be tested, followed by extraction of tumor and relevant normal tissues at several endpoints, and target inhibition is then assayed. This approach allows one to identify the best doses, schedules, and routes of administration for a compound that results in inhibition of the target in tumors and separates this from the question of anticancer efficacy. In vivo target inhibition studies can be completed in days or weeks compared to months for typical xenograft studies designed to evaluate tumor shrinkage or tumor growth delay, reducing costs and allowing more compounds to be evaluated in animals. Lilly Oncology is committed to the demonstration of in vivo target inhibition for all targeted agents we develop before proceeding to human clinical trials. In vivo efficacy studies can still provide valuable information about a compound prior to entering the clinic, but such studies will be conducted only after the dose and schedule necessary for complete target inhibition have been determined.

Clinical Development

The primary goal of phase I clinical trials of antineoplastic agents is to define the characteristics of a drug's acute toxicity in patients with advanced malignancies and to determine a dose and schedule for phase II studies that will be tolerable to most patients.

Cytotoxic agents have been investigated at or near their maximum tolerated dose (MTD), since few drugs have reliable biomarkers for biologic activity.[20] However, the goal of phase I studies of targeted agents should be to define the dose and schedule of a targeted agent that results in direct target inhibition in vivo.[19,21] This may be significantly lower than the MTD, especially for drugs that produce few toxicities. Studies could begin by defining the dose that results in serum concentrations consistent with target inhibition in preclinical models. Subsequently, direct target inhibition should ideally be confirmed in tumor biopsies or surgical specimens from patients after drug dosing.

If it is not possible to directly measure target inhibition in the relevant tissue, surrogate assays may be considered. These might include assays to measure the levels of target inhibition in another tissue such as peripheral blood mononuclear cells.[21] Alternatively, advanced imaging technologies, including positron emission tomography and magnetic resonance imaging, may provide a way to measure key biologic features of

the tumor closely linked to the mechanism of some targets such as markers of tumor angiogenesis.[19,22,23] These assays must be validated with respect to reproducibility and reliability and must be tightly correlated to direct target inhibition in preclinical models. Clinical development of the targeted agent should not proceed unless in vivo target inhibition can be achieved with acceptable toxicity. The tolerability of long-term dosing should be assessed if the agent is planned for long-term continuous administration.

Phase II trials are designed to confirm safety and to provide evidence of antitumor activity. If target-based therapies shrink tumors, standard trial designs can be used to evaluate these agents.[22] This was the case for 4 antibody therapies (trastuzumab [Herceptin, Genentech], rituximab [Rituxan, Genentech], imatinib [Gleevec, Novartis], and gefitinib [Iressa, AstraZeneca]) approved for the treatment of cancer in the last few years.[24] Many target-based drugs will only be growth inhibitory (cytostatic) and would not produce tumor shrinkage. This has led several groups to propose alternative trial designs that improve the chances of detecting clinically relevant activity of cytostatic agents.[22,25] Many of these designs incorporate time to progression (TTP) endpoints to assess single-agent activities, either as single-arm or randomized clinical trials.

Computational models predict that cytostatic agents could substantially increase the probability of cures when combined with cytotoxics by delaying development of resistance and slowing tumor regrowth between doses of cytotoxics.[26] In this context, a randomized study comparing the targeted agent plus a cytotoxic agent versus the cytotoxic agent alone could be used to demonstrate clinical efficacy.[22] This clinical trial design might be most applicable to patients with metastatic disease, where the cytotoxic agent is a current standard of care. Potential negative interactions between these agents would ideally be explored in preclinical models before beginning the trial and in a small number of patients in the clinic. However, such trials are not easy, especially if patients will be prescreened for expression and/or activation of the target before enrollment.

Translational Research and Biomarkers

While expression of the target in a patient's cancer cells is necessary, it is usually not sufficient to predict response. Gefitinib was only effective in about 9–19% of chemotherapy-refractory non–small-cell lung cancer (NSCLC) patients, and response did not seem to correlate with expression level of the target protein, the epidermal growth factor receptor (EGFR).[27,28] Recently, several groups identified mutations within the tyrosine kinase domain of EGFR associated with sensitivity of NSCLC to gefitinib.[29-31] Diagnostic tests based on sequencing the EGFR gene from DNA isolated from patient tumor samples are now being offered commercially to allow physicians to identify patients who are likely to benefit from gefitinib therapy.[32]

In addition to assays used to demonstrate in vivo target inhibition, the identification of biomarkers that predict response should be an integral part of the clinical development of targeted agents. These biomarkers can be used to stratify patients for clinical trials and to identify appropriate patients for treatment once the drug receives regulatory approval. Molecular characterization of tumors from patients treated with a targeted agent upon relapse or tumor progression can help identify new molecular targets for future drug development. When biomarkers exist for a significant number of anticancer therapies, the molecular profiling of a patient's tumor at diagnosis may become a routine approach that will allow physicians to personalize treatment to the patient to a much greater degree than is possible today.

Lilly Pipeline

Eli Lilly and Company has had a long commitment to developing effective cancer therapies, from the codiscovery of the vinca alkaloids in the late 1950s and marketing of vincristine and vinblastine in the early 1960s to the development and marketing of 2 cytotoxic agents, pemetrexed (Alimta) and gemcitabine (Gemzar), today.

- Gemcitabine hydrochloride is a nucleoside antimetabolite and is among the most studied chemotherapy agents in cancer as a result of its performance in some of the most common and most difficult to treat tumors. It is approved in the United States for the treatment of breast, pancreatic, and lung cancers.
- Pemetrexed disodium is an antifolate drug that inhibits thymidylate synthase, dihydrofolate reductase, and glycinamide ribonucleotide formyltransferase—all folate-dependent enzymes involved in the de novo biosynthesis of thymidine and purine nucleotides. It is currently approved in the United States for treatment of malignant pleural mesothelioma and second-line NSCLC.

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DRUG	CLASS	PRECLINICAL	PHASE I	PHASE II	PHASE III	MARKETED
Eukaryotic Initiation Factor 4E ASO	antisense therapy					
Transforming Growth Factor Beta Inhibitor	oral small molecule inhibitor					
Survivin ASO *	antisense therapy					
Serine/Threonine Kinase Inhibitor (Enzastaurin)	oral small molecule inhibitor					
P-Glycoprotein Inhibitor (Zosuquidar 3-hydrochloride)	small molecule inhibitor					
Gemzar (Gemcitabine hydrochloride)	nucleoside antimetabolite	BREAST, PANCREAS, LUNG				
Alimta (Pemetrexed disodium)	multi-targeted antifolate	MESOTHELIOMA, NON-SMALL-CELL LUNG				

Figure 1. Highlighted molecules in the Lilly pipeline.

ASO = antisense oligonucleotide.

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Lilly Oncology is continuing to develop innovative medicines for the treatment of cancer. Highlighted below are 2 molecules in Lilly's preclinical pipeline and 3 drugs in clinical trials that represent promising new targeted therapies (Figure 1):

- **eIF-4E ASO:** A second-generation antisense drug developed through a strategic alliance with Isis Pharmaceuticals that targets eukaryotic initiation factor 4E. eIF 4E is involved in the mRNA-ribosome binding step of eukaryotic protein synthesis and selectively enhances the translation of some mRNAs, including those of growth and survival factors.
- **TGFβ inhibitor:** An oral small-molecule inhibitor of TGFβ, a cytokine with diverse biologic activities, including promotion of tumor invasiveness, metastasis, and angiogenesis.
- **Survivin ASO:** Another second-generation antisense drug developed with Isis Pharmaceuticals that targets survivin. Survivin is a molecule that inhibits apoptosis and may allow progression of transformed cells through mitosis. Survivin ASO is currently in phase I clinical trials.
- **Enzastaurin:** An oral small-molecule inhibitor of serine-threonine kinases, particularly the protein kinase C β pathway. Enzastaurin has proapoptotic and antiangiogenic activities and is in phase II clinical trials in non-Hodgkin lymphoma and glioblastoma.
- **Zosuquidar 3-hydrochloride:** This is a selective and potent inhibitor of the adenosine triphosphate binding cassette transporter protein P-glycoprotein whose overexpression in some cancers confers a multidrug resistance phenotype. This drug is currently in phase III clinical trials in acute myeloid leukemia.

Summary

Developing effective molecularly targeted anticancer therapeutics relies on identifying the right target for the cancer, the right drug for the target, and the right patient for the drug. Target validation is critical, as is development of reliable, sensitive assays for identifying patients who will likely benefit from treatment. In mouse tumor models, direct evidence that a drug is "hitting the target" is required during preclinical development, although evidence of tumor shrinkage is no longer considered essential. The dose that results in complete target inhibition in human patients is used for further clinical evaluation if it is below the MTD. For cytostatic targeted agents, trials that incorporate TTP endpoints may be necessary to demonstrate a clinical benefit of drug treatment. Combination therapies of targeted agents and cytotoxics might be the most beneficial. In addition, oral chemotherapies may offer advantages to patients over intravenous drugs from a convenience and cost perspective.

Targeted therapies hold promise for the future of cancer treatment. Even if advanced cancers are not immediately curable, the possibility of turning cancer into a chronic condition with a good quality of life through continuous drug therapy might be almost as appealing.

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