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Meeting Report

In vivo Models for Experimental Therapeutics Relevant to Human Cancer

Miriam S. Gitler,[2] Edward A. Sausville,[1] Melinda Hollingshead,[1] and Robert Shoemaker[1]

[1]Developmental Therapeutics Program, National Cancer Institute, National Institutes of Health, Rockville, Maryland; and [2]NOVA Research Company, Bethesda, Maryland

The Developmental Therapeutics Program (DTP) of the National Cancer Institute (NCI) sponsored a symposium on *In vivo* Models for Experimental Therapeutics Relevant to Human Cancer on April 1, 2004, in Frederick, Maryland. About 50 scientists attended, with 8 invited speakers coming from Germany, the Netherlands, and various institutions in the United States. The meeting focused primarily on novel imaging approaches to cancer experimental therapeutics using animal models and pharmacodynamic (PD) end points. At the end of the presentations, a roundtable discussion was held in an attempt to reach consensus on a recommendation for optimal use of these modern imaging modalities. This report summarizes the meeting and provides a forum through which the cancer research community can provide input on the issues addressed in the roundtable discussion.

Dr. Robert Shoemaker (DTP, NCI, Bethesda, MD) opened the meeting with an overview of the evolution of animal models for experimental therapeutics, from transplantable syngeneic mouse tumor models to more complex models, such as chemically induced, transgenic, and spontaneous animal tumor models. Animal models for cancer drug development should include features relevant to human cancer, such as the representation of molecular targets, drug metabolism, pharmacokinetics, tissue distribution of a drug, anatomic relevance of tumor site, microenvironment, tumor natural history, angiogenesis, and metastasis. Similarly, critical in animal modeling are the end points for experimental therapeutic studies. Whereas change in tumor size is the most widely used end point, tumor frequency, survival, and change in tumor burden are other end points frequently used in this research area. Dr. Shoemaker observed that this meeting would focus mostly on new tumor imaging techniques that seem particularly relevant for measuring end points critical to experimental therapeutics.

Dr. Mike Cable (Xenogen Corp., Alameda, CA) described the advantages and limitations of biophotonic *in vivo* imaging, as well as the IVIS Imaging System developed by Xenogen. He explained that bioluminescent imaging is a powerful tumor cell labeling technique in which expression of a reporter gene (usually luciferase) is used for functional and tracer applications. In tracer applications, the reporter gene signal increases as cells divide and decreases as they die. Thus, the amount of light emitted at the surface is proportional to the number of cells expressing the reporter. Another useful property of bioluminescent imaging is that reporter expression can be tied to a particular promoter. Advantages of this approach include its high detection sensitivity due to extremely low backgrounds in control animals, relatively simple instrumentation requirements, and a wide range of applications. Bioluminescent imaging provides spatial and functional information. The IVIS Imaging System is a widely used workstation customized for quantitative animal imaging. The system is easy to operate and requires minimal training. Fluorescent imaging is another variant of optical imaging that can be quantitated using the IVIS Imaging System. However, fluorescent imaging is much less sensitive than bioluminescent imaging due to tissue autofluorescence. In closing, Dr. Cable observed that to measure absolute tumor size and location noninvasively, three-dimensional imaging is necessary because two-dimensional images provide only relative tumor growth curves. Tomographic optical imaging systems are under development at Xenogen.

Dr. Melinda Hollingshead (Biological Testing Branch, DTP, NCI) also focused on the use of bioluminescent imaging, describing DTP's experience with the IVIS Imaging System. She highlighted the advantages of using this system for measuring tumor burden, including increased sensitivity, decreased assay time, improved orthotopic model end points, and the opportunity to assess metastatic models, mechanistic studies, and micrometastatic disease. Cell lines engineered to express luciferase are needed for bioluminescent imaging. Because the amount of light emitted over a given amount of time varies by cell line, assessing the sensitivity of bioluminescent imaging with each cell line to be used in experimental therapeutics is recommended. DTP has successfully measured bioluminescence of luciferase-transfected tumor cell lines *in vivo* using the hollow-fiber assay and tumor xenografts, demonstrating in both cases a higher tumor detection sensitivity compared with other approaches. Antitumor activity of chemotherapeutic agents has also been measured effectively in animal models using bioluminescent imaging. Cautionary notes arose from observations that animal feed and other byproducts can emit a high luminescent signal and that frequent, repeated dosing with luciferin for reimaging purposes may have a cumulative effect on the luminescent signal. DTP is evaluating the pharmacology of luciferin after repeated dosing with this substrate. Other critical factors to consider in bioluminescent imaging include potential loss of tumorigenicity with transfection, the luciferin dose selected for imaging, the limited metastatic potential of many cell lines, a loss of linearity with increasing tumor volume, background signals masking small lesions, and the potential impact of therapeutic agents on luciferase activity and/or luciferin pharmacology.

Dr. Giovanni Melillo (Science Applications International Corporation–Frederick, NCI–Frederick, Frederick, MD) shared information on his *in vivo* models for the evaluation of hypoxia-inducible factor (HIF)-1–targeted therapeutics. HIF-1 is a good target for cancer therapeutics because it serves as a marker of tumor hypoxia; it is overexpressed in many human cancers, and HIF-1 expression is associated with angiogenesis, tumor progression, treatment failure, and poor prognosis. DTP has identified inhibitors of the HIF-1 pathway, specifically, a class of camptothecin analogs, including topotecan, that inhibit HIF-1–dependent induction of luciferase. Based on

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Note: E. Sausville is currently in the Greenbaum Cancer Center, University of Maryland School of Medicine, Baltimore, Maryland. M. Gitler is a science writer for NOVA Research Co.

Requests for reprints: Robert Shoemaker, NCI-Frederick, Building 440, Frederick, MD 21702. Phone: 301-846-6845; Fax: 301-846-6844; E-mail: shoemaker@dtpax2.ncifcrf.gov.

the *in vitro* findings, a U251–HIF-1–reactive element (HRE) tumor xenograft model has been developed in which the effects of chronic *versus* intermittent administration of topotecan on luciferase expression and several PD end points are being determined. Results thus far indicate that intermittent administration of topotecan has a more transient and less profound effect than chronic administration. Only chronic administration inhibits microvessel density and tumor growth. In addition, chronic dosing inhibits HIF-1α protein expression, as well as mRNA expression of vascular endothelial growth factor and phosphoglycerate kinase 1. Thus, sustained but not intermittent inhibition of HIF-1α may be associated with a biologically relevant response. These data support the use of this *in vivo* imaging model as a valuable tool for the evaluation of hypoxia-targeting agents; however, determination of PD end points at the tissue level is essential to better understand the molecular mechanism of targeted agents.

To close the morning session, Dr. Jos Jonkers (Netherlands Cancer Institute, Amsterdam, the Netherlands) expanded on the use of bioluminescent imaging for longitudinal monitoring of spontaneous tumor development in conditional mouse models of human cancer. Either transgenic or knockout mice can be used to generate conditional alleles of genes mutated in human cancer to induce spontaneous tumors. These models can be generated by recombinase-mediated gene switching. With the assistance of bioluminescent imaging, conditional models are being used to validate experimental therapeutic studies. The K-Ras^{v12} non–small-cell lung cancer model has been used to induce lung tumors by inoculation of Ad-Cre particles. A conditional luciferase transgenic mouse model (LucRep) has also been developed in which a stop element is removed by Cre, leading to expression of luciferase. Cross-breeding the two models generates a compound conditional mouse that can be used to longitudinally monitor lung tumors with bioluminescent imaging. Whether this model responds to therapeutic strategies used on human non–small-cell lung cancer remains to be determined. Single-agent (doxorubicin) and combinatorial [doxorubicin plus cyclin-dependent kinase (CDK) inhibitor] therapeutic interventions using a conditional pituitary tumor model have shown that the antitumor effect of doxorubicin closely resembles that observed in the clinical setting. This model has also served to demonstrate synergistic activity between doxorubicin and a CDK inhibitor in a spontaneous tumor model *in vivo*.

The afternoon session opened with a presentation by Dr. Umar Mahmood (Harvard Medical School, Boston, MA) on near-infrared fluorescence (NIRF) and magnetic resonance (MR) imaging in small animals. Optical imaging probes sensitive in the NIRF spectrum can be used for *in vivo* imaging of enzyme activity. Because proteases are overexpressed in many tumors, they represent an attractive target for antitumor imaging and therapeutic strategies. "Smart probes," which change their physical properties after specific molecular interaction with their targets, have been developed to detect specific protease activity (*e.g.,* cathepsin D) in tumors. The effects of therapeutic agents whose targets are at the protein level (*e.g.,* matrix metalloproteinase 2 inhibitors) as well as the "aggressiveness" of tumors can be optically imaged with NIRF probes. The intensity of the fluorescent signal is proportional to the protease activity, and this can be correlated with the level of dysplasia. Compared with other imaging modalities, NIRF allows multichannel imaging at different wavelengths in the same animal; thus, expression of two different genes can be monitored simultaneously. Small animal fluorescent endoscopy has been developed to study abdominal organs and colon cancer.

Another optical imaging modality being used for detection of deep tumors and experimental therapeutics is tomographic NIRF imaging, in which high spatial resolution appears in areas of protease activity and fluorescence correlates with *ex vivo* histology. MR imaging is being used for small animal phenotyping, receptor imaging, and cell tracking, as well as for detecting angiogenesis, apoptosis, and marker genes in gene therapy. Lastly, optical NIRF imaging has been combined with MR imaging to yield highly detailed molecular and anatomic information in small animals. Dr. Mahmood stressed the potential translation of these imaging modalities to the clinical setting.

Dr. Lily Wu (University of California Los Angeles, Los Angeles, CA) addressed the use of gene expression-based imaging coupled with gene therapy in advanced human prostate cancer xenograft models. The expression of a specific reporter or therapeutic gene can be targeted to tumor cells by inserting a specific promoter into an adenoviral vector. Targeted gene expression can then be monitored *in vivo* using noninvasive optical imaging techniques, allowing detection of diseased tissue and accurate monitoring of the kinetics and levels of transgene expression. Dr. Wu has used two approaches to enhance the activity of the prostate-specific antigen promoter to drive the expression of the firefly luciferase reporter gene. In the first approach, multimers of androgen-responsive elements are incorporated into the construct; in the second approach, a two-step transcriptional amplification (TSTA) system is used, in which a modified prostate-specific antigen promoter drives a potent synthetic transcriptional activator, which in turn activates the expression of a reporter or therapeutic gene. Using the first approach, targeted luciferase expression has been identified in distant metastatic LAPC prostate cancer cells in animals. Expression of firefly luciferase has been amplified in LAPC prostate cancer cells with the TSTA approach. The TSTA system has also been used for targeted expression of therapeutic genes (*e.g.,* herpes simplex virus-thymidine kinase). Positron emission tomography (PET) imaging has been adapted to gene-based imaging in small animals using the herpes simplex virus-thymidine kinase reporter gene. Other applications of TSTA gene expression imaging include the monitoring of acute androgen withdrawal therapy and real-time imaging of androgen receptor function. This approach appears to be feasible also in androgen-independent and metastatic prostate cancer.

Professor Heinz-Herbert Fiebig (University of Freiburg, Freiburg, Germany) contributed to the understanding of *in vivo* drug–target interactions by presenting an overview of the characterization of molecular targets in the cell lines constituting the Freiburg collection of human tumor xenografts. As an example of drug–target interactions, he described the mechanism of action of 17-demethoxy-17-*N,N*-dimethylaminoethylamino-geldanamycin (17-DMAG), a geldanamycin derivative. Heat shock proteins (HSPs) are the molecular target of 17-DMAG. Whereas 17-DMAG depletes the HSP90 and client proteins such as c-erbB2/HER2, CDK4, and Akt, it induces HSP70, causing cell cycle arrest and apoptosis. The compound is more active in cell lines expressing high levels of HSP90 than in those expressing low levels of HSP90. A second example of drug–target interactions was provided with respect to halofuginone, a quinoxaline alkaloid. The agent inhibits collagen types I and II, matrix metalloproteinase 2, and vascular endothelial growth factor and reduces metastasis and angiogenesis. Halofuginone is currently under phase I clinical evaluation by the European Organization for Research and Treatment of Cancer. Studies with tumor xenograft models have shown that the antitumor activity of halofuginone correlates with a decrease in the protein expression of collagen type I, as well as decreased microvessel density. Molecular characterization of the Freiburg xenograft collection has allowed the selection of tumors overexpressing the target of interest for *in vitro* and *in vivo* experimental therapeutic studies.

To complete the afternoon session, Dr. Allan Johnson (Duke Medical Center, Durham, NC) provided an overview on the use of small animal microscopy for cancer research. He illustrated challenges with small animal imaging hardware and provided examples of microradiography, microcomputed tomography, and MR microscopy. Monitoring the animal's physiologic state and stability is critical in small

animal imaging. Duke's Center for *In vivo* Microscopy has integrated the animal monitoring signals with the imager, so there is feedback between the animal and the scanner. Another challenge is exemplified by microradiography, in which the exposure time for imaging blood vessels needs to be significantly shorter for a small animal than for a human. Duke investigators have modified a conventional angiographic system to overcome this problem. Microradiography is being used for cancer therapeutics, especially for longitudinal studies of vascular development. Another X-ray microscopy imaging modality is microcomputed tomography, which allows imaging of solid tumors at 50-μm resolution. Dr. Johnson elaborated on the ongoing adaptation to small animal experimentation of state-of-the-art clinical MR microscopy developed in collaboration with General Electric. He also highlighted the advantages of using MR histology: tissue is not damaged; soft tissue contrast is excellent; it is inherently three-dimensional; and data sets are inherently digital. Because of the high price of high-resolution MR magnets, other strategies have been developed to improve resolution, including the use of paramagnetic contrast agents and formaldehyde for tissue perfusion and fixation. MRPath is commercializing the microscopy imaging technologies developed at Duke through its Visible Mouse Project. The project will provide a new tool for studying the morphology of normal and genetically altered mice and the capability of accessing and sharing image data sets through the internet.

Roundtable Discussion

To stimulate discussion, Dr. Edward A. Sausville (DTP, NCI) presented the panel of invited speakers with a preliminary proposal for a new NCI initiative on how to accelerate the transition of drug development from the preclinical to the clinical setting by testing, in phase I trials, new agents in terminal patients with no alternative form of therapy of known benefit. Current United States Food and Drug Administration regulations for entry of a new molecule into early clinical trials focus on toxicity evoked in test species. However, with the advent of targeted therapies, molecularly targeted agents may act by targeting receptors that might not convey overt toxicity or might evoke cytotoxicity as a response to binding a receptor. Thus, safety testing requirements need to be revisited. On such a basis, a new path for cancer drug development could be charted in which phase I clinical trials would be driven not by the occurrence of toxicity but rather by predictable pharmacokinetic (PK) or PD end points. With this in mind, the proposal for the new initiative is timely because information in humans is needed earlier rather than later, successful PK with PD markers would greatly increase the value of the product to potential developers, and PK data would allow actual dose-escalation schemes to be modified as needed. Cautious clinical dose escalation would be retained, but it would be tied in real time to PK/PD assessments. Following phase I, but before committing to full-scale development, a complete toxicology evaluation would be conducted, taking into consideration patient PK data for study design and analysis. Animal and human "relevant" compartment information (*e.g.,* bone marrow) would be correlated at efficacious concentrations, and safety testing would be tapered to exploring single- and limited multiple-dose PK in a range of species. The starting dose would be approximately one-sixth lower than the dose required to achieve target modulation, measured as an area under the curve or ED_{50} (50% effective dose) in assessable compartments; toxicity would be observed at "*n*-fold" the desired area under the curve or ED_{50} of the species studied, rather than seeking to apply dose escalation to cause toxicity. One animal species (normally rat) would be selected to explore toxicity determination.

Implementation of this approach was exemplified with the proteasome inhibitor PS-341, whose starting dose and highest dose in its initial phase I trial were based on the ED_{50} that caused an effect in a surrogate compartment. The questions then posed to the panel were as follows: How can the novel imaging approaches presented at this symposium be used to provide the required early PD information to implement the proposed PK/PD paradigm? Which of the imaging technologies is at the most advanced stage for development along with the PK/PD paradigm? Which of the imaging approaches is most amenable to quality control and quality assurance? Is there one cancer type that is more suitable than others for consideration using these technologies?

Panelists discussed the value of imaging nonspecific end points in animal models at a relatively early time in the natural history of a tumor in the absence of a clear mechanistic pathway for which a specific end point could be measured. Some panelists argued that the less specific an early biomarker, the more useful it is because it can be applied to a larger number of drug candidates, many of which have no known clear pathway. Luciferase was cited as one such broadly applicable readout that provides direct correlation with tumor mass. Another panelist underscored the importance of making a distinction between a "mechanistic" and a "toxicity" end point, maintaining that whereas a toxicity end point could provide a readout of the biological activity of the drug that would drive the toxicity studies, it does not necessarily correlate with the therapeutic effect of the drug. This panelist contended that a mechanistic end point is more important for driving the rational development of a drug and that the biochemical data supporting such a mechanism of action are critical for designing the *in vivo* model. The suggestion was made to couple two or more imaging modalities so that both apoptosis and mechanistic pathway end points can be measured.

In an attempt to answer the first questions posed, a panelist ranked the imaging modalities for the purpose of initial screening according to sensitivity, from worst to best, as computed tomography, MR, PET, and optical imaging. Optical and PET imaging are comparable in the sense that a reporter system could be adapted to PET to provide mechanistic end points, but optical imaging is more cost-effective.

With respect to quality control, remarks were made in relation to bioluminescent imaging, mainly, that the luminescent signal does not always correlate with tumor mass, and therefore a better understanding of the impact *in vitro* of experimental agents on the microenvironment and on the imaging system itself is needed before this technology can be widely applied. It was debated whether this modality is capable of accurately defining a dose and schedule that could be translated into the clinical setting. Assuming that the ideal agent exists for which there are corroborating bioluminescent imaging-derived mechanistic data, this modality would seem to be reliable for defining a clinical dose. Reproducibility, however, was another issue of contention because it was argued that whereas this imaging modality could be reproducible for end points such as tumor mass, it would vary for mechanistic end points based on cell type and pathway. Although a consensus recommendation was not attained, there was a collective feeling that NIRF imaging of proteases might constitute an appropriate early biomarker that could be used generically as a surrogate end point (*i.e.,* apoptosis) to define a clinically useful outcome.

DTP would like the cancer research community to react to the issues addressed at the roundtable discussion and provide the NCI with feedback.