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Preclinical Models of Immune oncology for CART-cell Therapeutics Development

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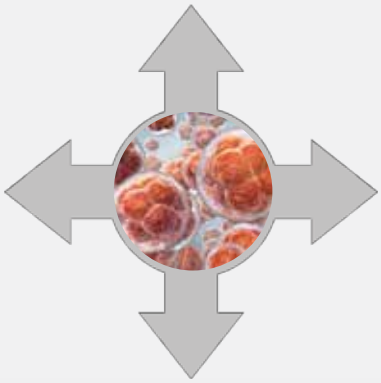
(Sources: Public information and Case studies from Aragen)

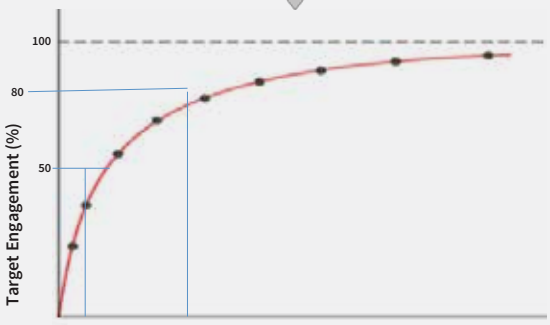
HUMAN CELL LINE
XENOGRAFT MODELS

MTD STUDIES



SYNGENEIC/ XENOGRAFT/
ORTHOTPIC/ PDX
TRANSGENIC, GEMM
TUMOR MODELS

BIOANALYSES / PK-PD
CORRELATION



IMMUNOONCOLOGY
MODELS-CAR T-CELL
THERAPEUTICS

EXECUTIVE SUMMARY

According to the American cancer Society (ACS) [1], in 2021, 1.9 million new cancer cases diagnosed, and 608,570 cancer deaths reported in the United States. Treatment for cancer is complicated. The mainstream anticancer drugs, including chemotherapeutics and targeted drugs are not completely curative and cause unwanted side effects. Novel small molecule inhibitors targeting oncogenes, antibodies, engineered CAR T cell therapeutics, and antibody drug conjugates (ADCs) targeting host immune response molecules were approved recently. In the future, more novel targeted anticancer drugs that overcome drug resistance and cause minimum side effects continue to be developed through preclinical IND-enabling studies and consequent clinical trials. Therefore, urgent attempt is required to develop new therapies aided by effective and clinically relevant IND-enabling preclinical oncology models. This white paper discusses several preclinical IND-enabling oncology case studies performed over 10-years by Aragen's scientists. For more information and to connect with our team, visit: Oncology - Aragen Life Sciences

Key words: Preclinical oncology animal models, in vivo and in vitro tumor models, preclinical-immunooncology, clinical oncology, pharmacology, toxicology, CAR T-Cell Therapy, Immune System

Background

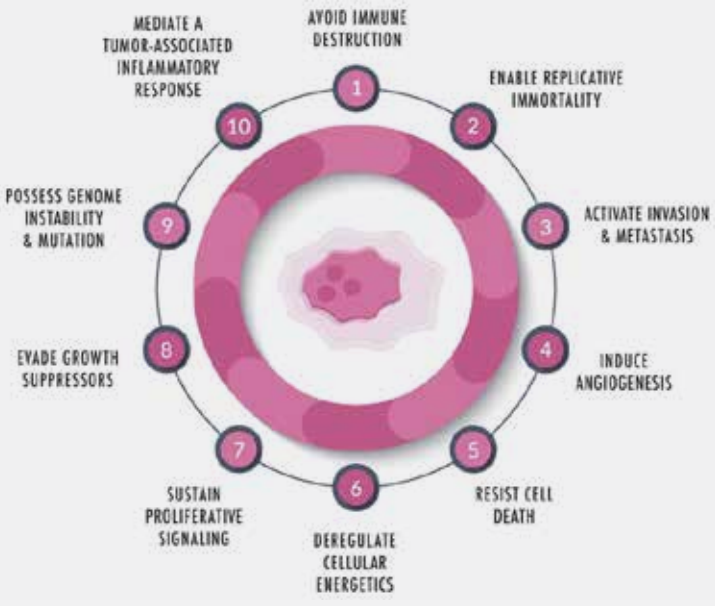
According the NCI (Cancer Statistics - NCI) [2], an estimated 1,806,590 new cases of cancer was diagnosed in the United States in 2020, and 606,520 succumb to cancer. The most common cancers are breast cancer, lung and bronchus cancer, prostate cancer, colon and rectum cancer, melanoma of the skin, bladder cancer, non-Hodgkin lymphoma, kidney and renal pelvis cancer, endometrial cancer, leukemia, pancreatic cancer, thyroid cancer, and liver cancer. per ASCO.ORG [3], brain tumors account for 85% to 90% of all primary central nervous system (CNS) tumors. In 2022 an estimated 25,050 adults in the United States will be diagnosed with brain tumors. Metastatic brain tumors account for an estimated 200,000 new cases of brain metastases in the U.S. every year (Yalemedicine.org) [4].

Understanding the mechanism of cancer progression for developing new Anticancer drugs

Pathogenesis of cancer is complex due to different types of cancers- their etiology, biology and the human organs that are affected. Cancer is a chronic disease characterized by uncontrolled cell growth.[5] Cancer cells typically go through four stages – initiation, proliferation, invasion, and metastasis (Figure). There are over 100 different types of cancer, and each is classified by the type of cells that are initially affected. Cancer cells divide uncontrollably to form lumps or masses of tissue called tumors that can grow and interfere with several bodily functions.

Targeting Cancer Cell signaling to Counter Growth

Cell signaling allows intercommunication of individual cells to everchanging extracellular signals originating from extracellular matrix, hormones, or growth factors. These extracellular signals activate intracellular signals to facilitate normal cell functions such as proliferation or differentiation, migration, invasion or undergo apoptosis (programmed cell death) or necrotic cell death. However, neoplastic cells possess dysfunctional cell signaling process called signal transduction or transmembrane signaling. This leads to abnormal control of basic cellular activities via faulty responses caused by uncoordinated communications across the cell. Cancer cells acquire the ability to hijack normal cell



function and pave way for abnormal cell functions as described by Hanahan and Weinberg [7]. See Figure [Adapted from "The 10 hallmarks of cancer [7]". Many researchers have investigated the molecular basis of tumor initiation, translation, progression, and metastasis to distant systemic organs including brain. Specifically, the role of channels including potassium channels in cell adhesion, migration and invasion is recently revealed. For example, the calcium-activated voltage-sensitive potassium (BKCa) channels interact with a variety of proteins both at the plasma membrane and with intracellular organelles including the endoplasmic reticulum, nucleus, and mitochondria. Splice variants of BKCa were shown to be involved in glioma progression and breast cancer metastasis to brain [8].

Targeting Tumor Vasculature

Vascular endothelial growth factor receptors (VEGFRs) and vascular growth Factor (VEGF) are involved in neovascularization in cancers. Deregulated VEGF expression from cancer cells contributes to the development of solid tumors by promoting tumor angiogenesis. Consequently, inhibition of VEGF signaling abrogates the development of a wide variety of tumors. The second-generation multitargeted tyrosine kinase inhibitor targets VEGFR, platelet-derived growth factor receptor, and c-kit as key proteins responsible for tumor growth and survival. Pazopanib exhibits good potency against all the human VEGFRs and closely relate to tyrosine receptor kinases *in vitro* and demonstrates antitumor activity in several human tumor xenografts. Therefore VEGFR-pathway-based phenotypic drug screening succeeded in discovery of first-in-class anti-cancer drugs. Recently, immunooncology has emerged as main strategy to fight against cancer, supported by better understanding of how cancer cell evades the natural immune response. Advanced technologies such as next-generation sequencing (NGS) made easier to accurately study immunotherapy response factors, discover biomarkers, and apply genomics to personalized cancer immunotherapy. For example, NGS helped identify pathways that are activated in the tumor environment and processes promoting cancer cell proliferation, survival, invasion, and metastasis. These developments resulted in rigorous effort for targeting immune response markers as developing new and effective targeted anti-cancer treatment strategies.

Preclinical testing of anti-cancer therapeutics in clinically relevant animal models

A wide range of rodent models are developed to discover and develop anticancer drugs, from traditional subcutaneous tumor models to genetically engineered mouse that spontaneously give rise to tumors [5,6]. These models allow scientists to consider the best time to use the models, along with practical applications and shortcomings. Most importantly, these models will reflect the underlying cancer biology and guide us in predicting anticancer drug efficacy in the clinic. Therefore, the selection of appropriate preclinical models and target clinically relevant biomarkers very early in the discovery project provides a clear benefit, as it helps expedite the drug development process.

Aragen offers a diverse range of preclinical oncology models and services with client-specific customized study design. These include human xenograft tumor models as well as the more complex sub-renal capsule, patient-derived xenograft (PDX), and syngeneic tumor models. Xenograft models are extensively used in IND-enabling studies for evaluation of NCEs and NBEs as potential anticancer agents. Our scientists have developed and validated several xenograft models in Ncr Nu/Nu, NOD-SCID and SCID-Beige mice. We will customize and utilize any human cell line of your interest to conduct *in vivo* proof-of concept studies. Syngeneic models allow scientists to test anticancer agents in an immunocompetent system, although it may not entirely reflect the human immune competency. However, with intact immune system, syngeneic models are pertinent for evaluating immunologically based targeted therapies alone or in combination. We monitor PK/PD correlations using plasma following test article administration, tumor response, changes in tumor volume and weight, as well as studying genomic, proteomic and metabolomic biomarkers.

Immunooncology (IO) and CAR T-Cell therapeutics

Successful preclinical cancer research study outcome entirely depends on selection of an appropriate animal model. Depending on the type of cancer, a researcher will have to consider what type of rodent (Mouse or Rat), syngeneic (immunocompetent) or xenograft (immunocompromised), location of the tumor, type of tumor cells to grow and where to inject and grow (sub cutaneous, intraperitoneal, brain, metastases (lymph nodes, lung, breast, brain etc.). Targeting a biomarker (enzyme, receptor, oncoprotein, oncogene etc. with small molecule, large molecule (proteins, modified proteins, DNA, RNA, peptides, gene therapy (AAV assisted), molecule produced or expressed by host immune system in response to antigens/ vaccines is a standard strategy in preclinical anticancer drug development.

Pre-clinical study to show the ability of a test article to activate the innate immune response in patients, requires human tumor and the immune cells in a mouse with functioning immune system (humanized mouse). Such humanized mice models helped researchers to identify mechanisms of action and targets for therapeutic interventions. Immunooncology research is more focused on the checkpoint inhibitors, CAR-T cells, modified TCR T cells, and vaccines in various forms. Due to extensive IO research, more effective and targeted treatment strategies for cancer have evolved in recent years. The approval and use of chimeric antigen receptor (CAR) T-Cell therapeutics in difficult-to-treat cancers has brought hope to millions of patients and oncologists globally. In particular, the success of CART-cell therapy in hematologic malignancies renewed efforts to use this technology against solid tumors as well. However, this strategy has had several challenges, including targeted delivery, penetration of therapeutics to tumor cells, immunogenicity, short- and long-term toxicity and safety, and efficacy of CART-cells in humans. To address the challenges above, several animal models have been utilized to screen potential CART-cell therapeutics as part of the IND-enabling process.

In addition, bispecific antibodies have emerged as a new class of therapeutic agents designed to simultaneously bind to patient's T cells and to tumor cells, inducing T-cell-mediated cytotoxicity of tumor cells. Humanized models with human peripheral mononuclear blood cells (PBMCs) in combination with a variety of xenograft models, are the most frequently used *in-vivo* platform for short-term screening of novel compounds. Full hematopoietic stem cell (CD34+) reconstituted models combined with genetically modified immunodeficient strains are utilized for long term screening of T-cell modulators, natural killer (NK) cell modulators, and other agents that induce antibody dependent cell cytotoxicity (ADCC), as well as various categories of immune checkpoint inhibitors and agonists.

Expertise at Aragen

A team of experienced scientists and skilled *in vivo* research associates will support pre-clinical research from study design through execution as follows:

- Ensuring the successful intravenous adoptive transfer of CART-cells or human PBMCs into a mouse.
- Experience in performing in vivo CART therapeutic evaluations of both liquid and solid tumors (Figure 1).
- Experience in using cryopreserved CART cells or human PBMCs with careful thawing and resuspension prior to the adoptive transfer (8).
- Experience in performing a wide range of cell line derived xenograft in humanized mouse models with successful identification of efficacy to support preparing IND program.
- Utilization of our bioluminescent imaging system to monitor tumor progressions in living animals (Figure 2).
- *Ex vivo* supports including detection of tumor-specific or associated antigens on the surface of cancer cells by FACS, pre-screening of the PBMCs both *in vitro* or *in vivo* to reduce donor variability and to ensure consistency replicate studies by using the same donors, monitoring of CART cells in the host, and screening of human PBMCs engraftment (Figure 3).

Our research facilities are in Morgan Hill, CA and we can accommodate same-day or second-day domestic shipping of your temperature-sensitive therapeutics and biological samples.

Validated Immunooncology studies

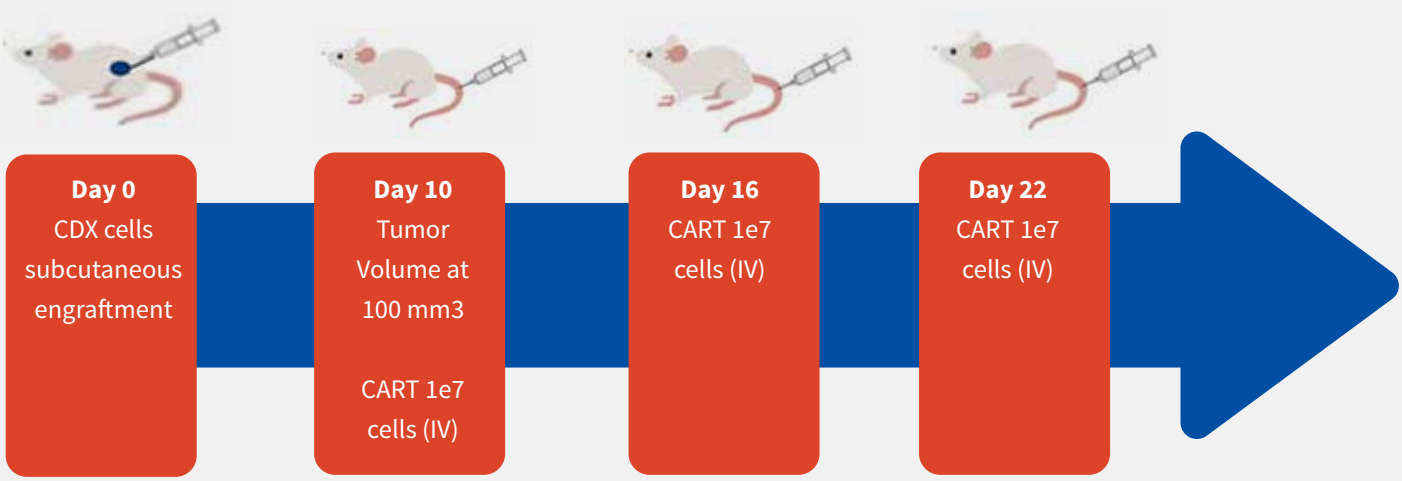
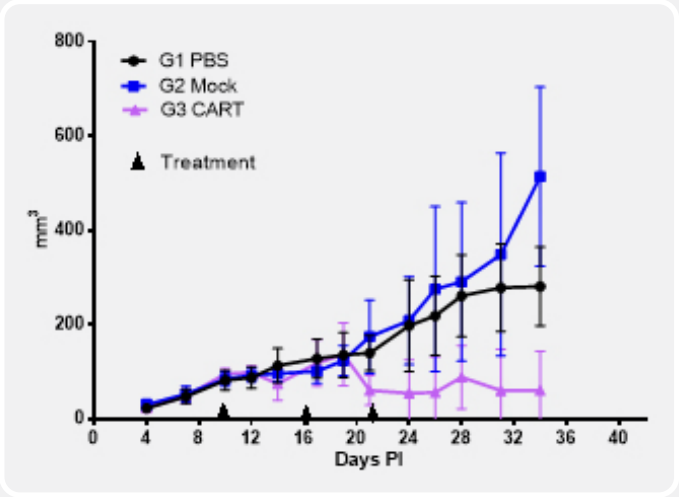


Figure 1. Representative study design for evaluating CART therapeutics

- Blood sampling for monitoring circulating CART
- Tumor volume follow up
- Clinical observations and body weight measurement

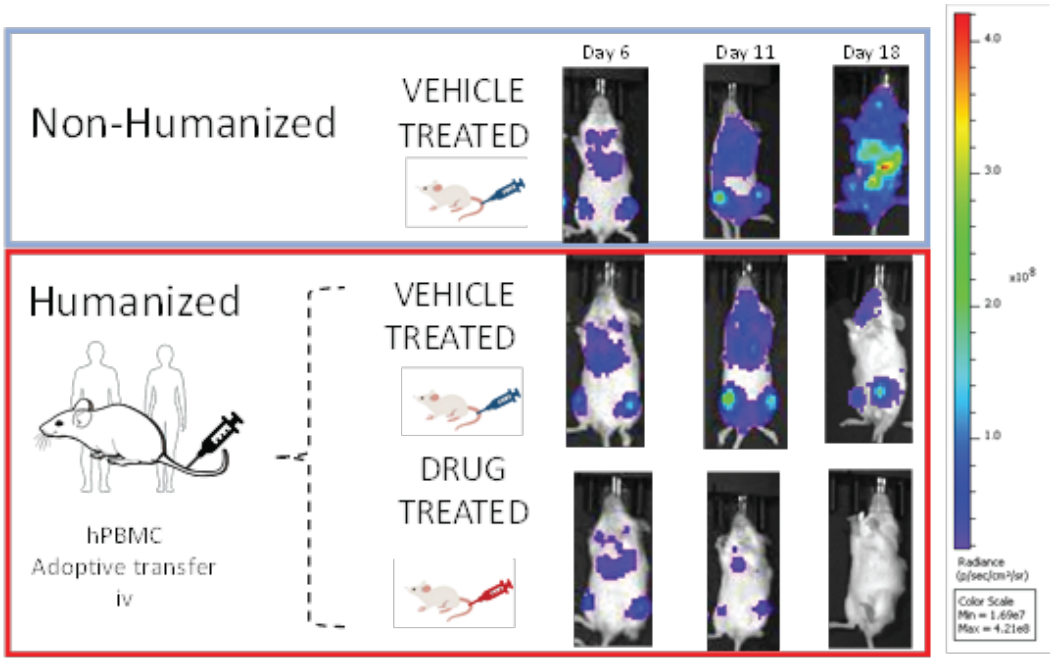


Humanized animal models for evaluating immunotherapeutics

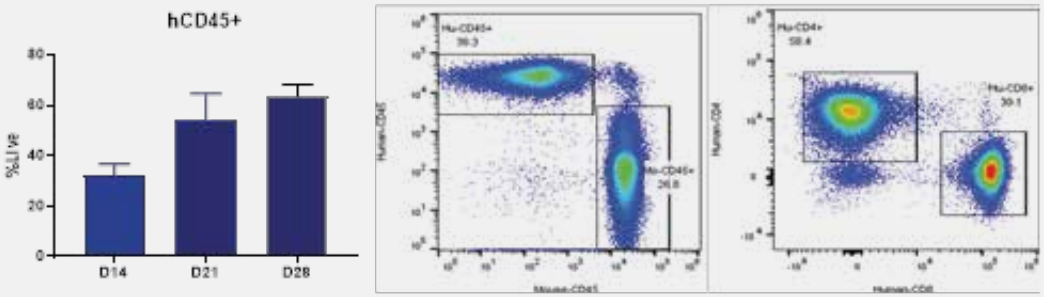
Immunotherapeutics are shown to restore and stimulate human immune response to slow down tumor growth and/or destroy cancer cells. Unfortunately, only a partial clinical response rate observed in few patients.

Humanized mouse models are routinely used to develop personalized precision drug candidates to treat cancer. This model assumes significance as an important strategy for evaluating novel cancer immunotherapeutics *in vivo*.

Case study 1: Ventral view images of mice after inoculation (6, 11 and 18 days) of Raji-Fluc cells both in non-humanized and humanized models.

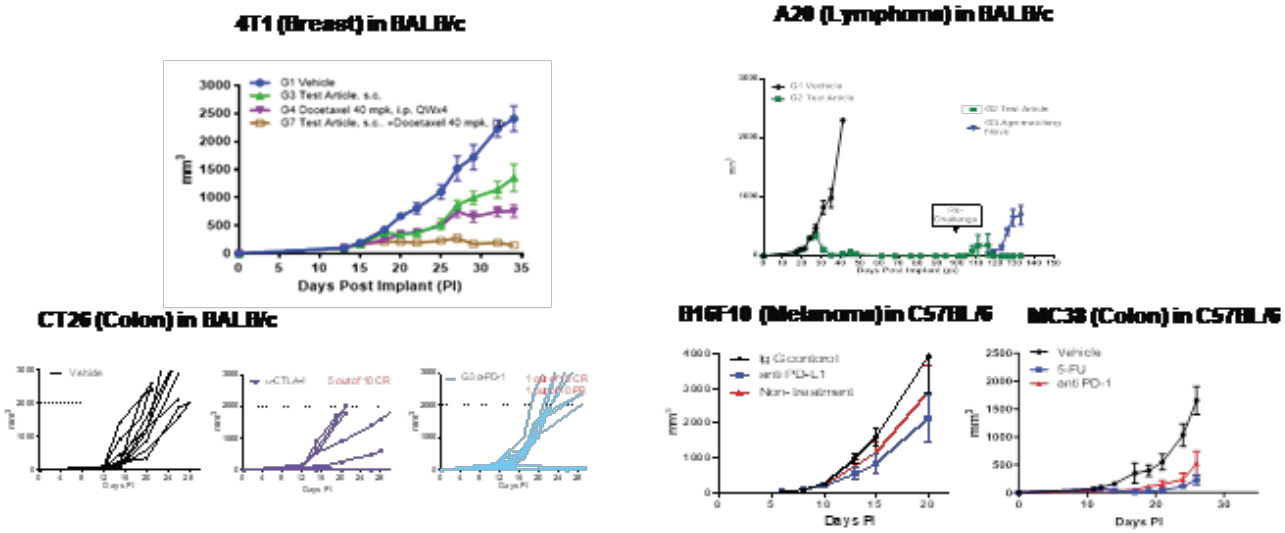


Case study 2: Engraftment of human PBMCs

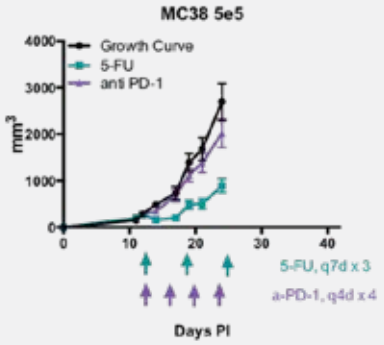


Bar graphs of CD45+ engraftment after hPBMC adoptive transfer. The leukocyte marker CD45+ and T-cell markers CD4+ and CD8+ measured from peripheral blood on 14, 18 and 21 days.

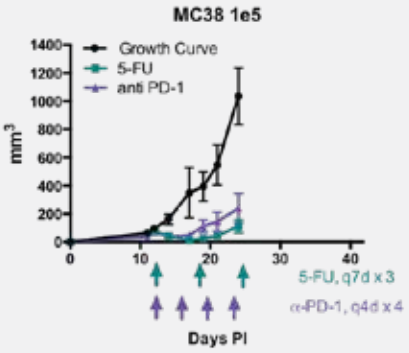
Case study 3: Various Syngeneic Tumors Developed in Mice to evaluate Anti-cancer molecules

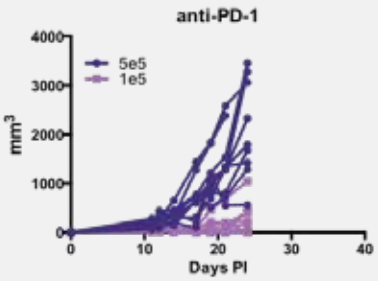


Case Study 4: Evaluating Immune Check Point Inhibitors: 5-FU or Anti-PD-1 (RMP1-14) Therapies in Syngeneic Mouse Models (MC38)



Treatment initiated at tumor size 150 mm3





Treatment initiated at tumor size 60 mm3

Patient-derived tumor xenografts (PDX) in preclinical studies

Although majority of anticancer drugs have entered the clinical development after extensive preclinical pharmacology evaluation criteria, only a handful of drug candidates have been successful in clinics. Failure to use a clinically relevant *in vivo* preclinical pharmacology models is often cited as a main reason for the lack of clinical effectiveness in humans. Hence, pharmacology studies conducted with patient derived xenograft (PDX) tumors appears to improve the predictiveness of preclinical efficacy models for clinical success of anticancer drugs.

Recent studies have shown the superiority of preclinical models using PDX patient-derived xenograft (PDX) tumors over cell line derived xenograft (CDX) models, especially when clinically relevant doses were administered. Importantly, PDX models more closely recapitulate the heterogeneity of patient tumors and maintain the molecular, genetic, and histological properties of original tumors, especially in the early stages of sequential passaging in mice. This is key to evaluate tumor response or resistance to therapeutic intervention. Most notably, PDX models allow target or biomarker identification that can identify patients who would benefit from targeted anticancer drugs.

Transgenic Mouse Models for Cancer Research

Transgenic mouse is created by the introduction of new and functional genes into the germ line. These genetically engineered mouse model (GEMM) is used to explore and establish the casual link between candidate cancer genes and carcinogenesis. Additionally, the GEMM helps to develop and test new targeted therapies more efficiently than the conventional syngeneic and xenograft mouse models. Other types of mouse models are developed wherein oncogenes are constitutively or conditionally expressed and tumor-suppressor genes silenced by DNA constructs and siRNA. Such mouse models are extremely useful in studying mechanism of carcinogenesis and tumor resistance and pathogenesis.

Recently, the clustered regularly interspaced short palindromic repeats (CRISPR)-based genome editing approach contributed to develop CRISPR/Cas9-based transgenic models that can mimic wide spectrum of mutations found in human cancers. For example, a humanized mouse xenograft model that accept PDX and CD34+ cells were developed to study human tumor heterogeneity, the tumor microenvironment, and interaction between the tumor and human immune cells. This novel technology enabled personalized cancer therapy and better patient survival and quality of life.

Genetically engineered mouse models ((GEMMs) in Cancer Research

GEMMs are superior to the implantation models because GEMMs develop *de novo* tumors in a natural immune-proficient microenvironment. Therefore, the tumors in GEMMs closely mimic human cancers with respect to histopathology and molecular features as they spontaneously progress toward metastatic disease. Therefore, GEMMs are indispensable for preclinical studies to validate candidate cancer genes and drug targets, assess response or resistance to treatment. This model is well suited to evaluate therapy efficacy and dissect the impact of the tumor microenvironment and host immune response. Furthermore, the preclinical cancer intervention studies in GEMMs might be readily aligned with clinical studies in patients, giving much needed impetus to hasten therapeutic candidates and their translation into the clinic.

Multiplex Immunophenotyping

Aragen scientists have performed immunophenotyping in different cell lines using Attune (14 color FACS) as shown in the following Table. Different subsets of immune cells were tagged, and different T cell counts were measured. Immunophenotyping couples specific antibody to a fluorescent compound and helps in measuring specific protein expression within a cell population. The protein expressed in certain cells are used to identify and categorize the population of tagged cells. It is often used to measure CD4-T cell counts in specific immunodeficiency disorders and immune-related diseases. It is also used to detect the presence or absence of biomarkers of cancer in cancer cells to predict their severity in forming tumors and to study targeted drug effects.

Human Cell Line	Origin of Tumor
SKOV3	Ovarian cancer
Bx PC3	Pancreas
FaDu	Cervical Carcinoma
786-O	Renal Carcinoma
Caki-1	Renal Carcinoma
Luciferase Tagged Cell Lines	
MDA-MB-231-Luc	Breast
MCF-7 Luc	Breast
SKOV3-Luc	Ovarian cancer
MKN-1-Luc	Gastric cancer
Mia Paca-2-Rluc*	Pancreas
786-O-Luc	Renal Carcinoma
Caki-1-Luc	Renal Carcinoma
A673-Luc	Muscle Ewing's sarcoma
Raji-Luc	Burkitt's lymphoma

Tumor Imaging

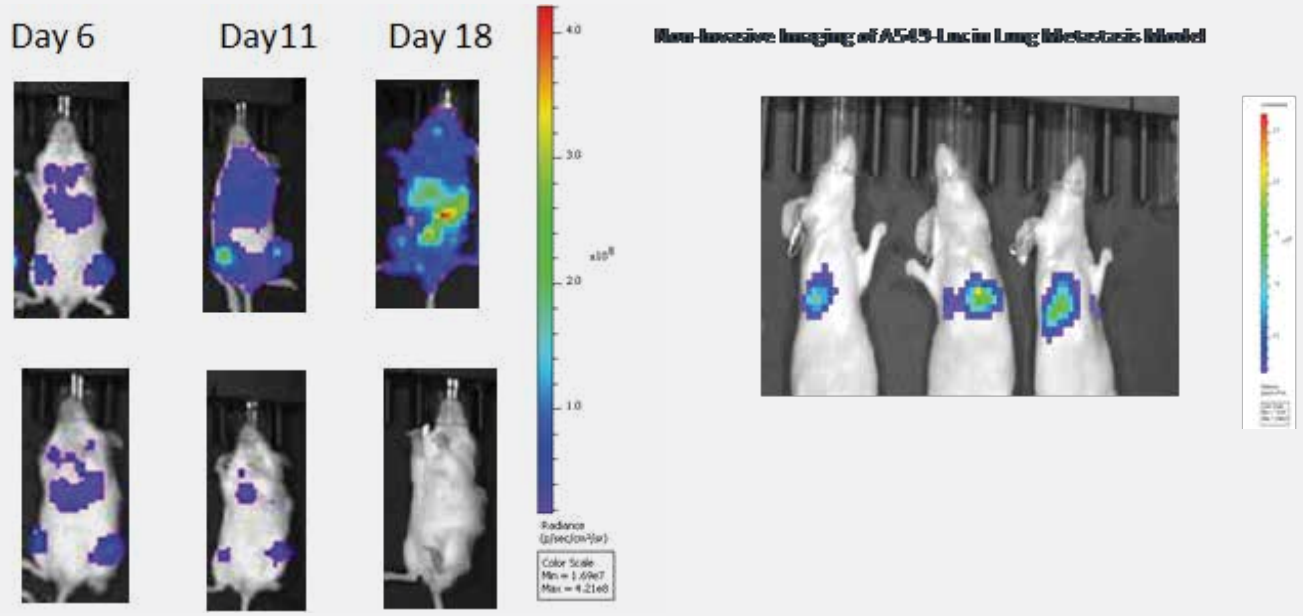
Imaging is a critical tool for non-invasive assessment of biological and biochemical processes in living subjects. IND-enabling studies of any new therapeutics is incomplete without relevant images capturing targeted biological and biochemical processes. Imaging tools help us decide or select drug candidates that most likely succeed in a vigorous clinical trial process. Here, we present few preclinical oncology case studies, for example breast and ovarian cancer models that show key role of molecular imaging in drug development process.

Imaging is part of most protocols for conducting preclinical and clinical studies of various anticancer drugs since it provides morphological, structural, metabolic, and functional information of drug-target interactions. In clinics, biomedical imaging serves as the foundation of comprehensive cancer care, offering benefits such as non-invasive real-time monitoring of biological and pathological processes.

Aragen's competences as a leader in preclinical oncology services is built on more than a decade of in anticancer drug development using appropriate animal models and precision molecular imaging tools. Aragen scientific team will design, customize, and execute the preclinical oncology studies with major input form the clients and partners. Our entire teamwork in tandem with the clients to ensure and expedite workflow in a cost-effective and timely manner. Our state-of- the-art imaging facilities include Multi species optical and X-ray imaging, advanced *in-vivo* fluorescent and bioluminescent imaging and Multiplex Immunophenotyping. We show case a few preclinical oncology case studies using Syngeneic, Xenografts, Orthotopic and metastatic tumor models, which highlight the key role of molecular imaging in the drug development process.

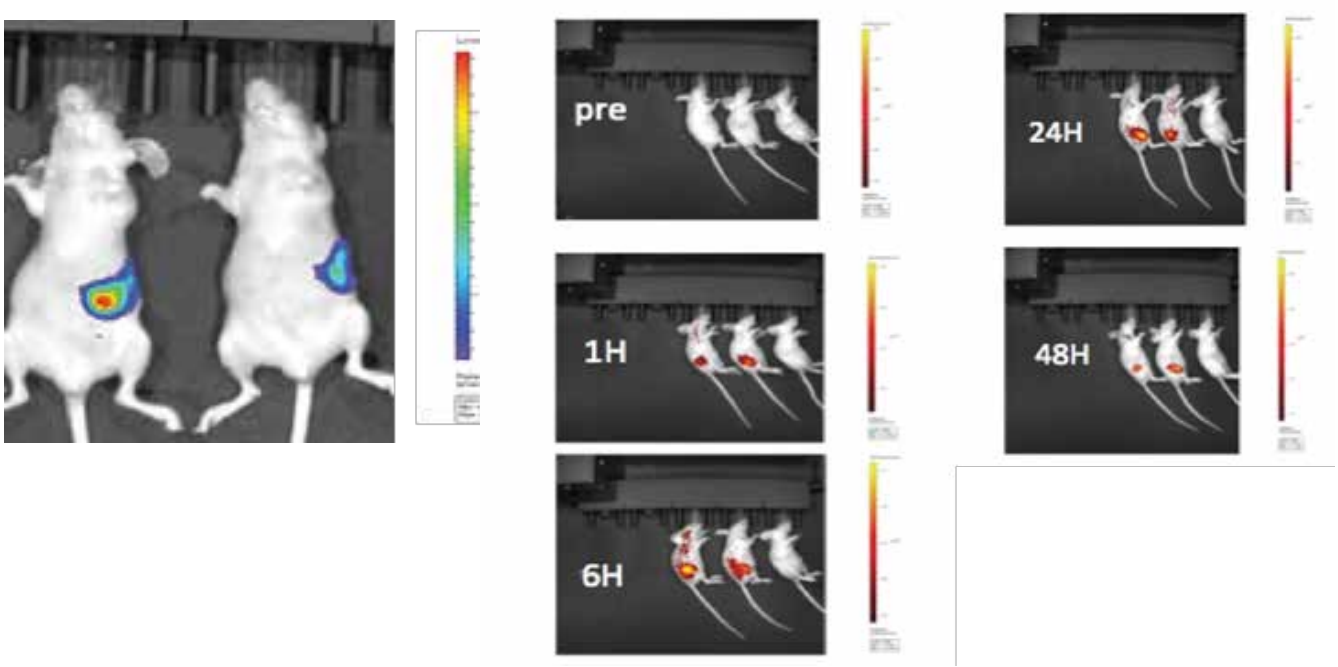
Case Study 5: Optical and X-ray Imaging performed on Mice with implanted with different types of cancer cells.

PE IVIS XRMS System for multi-species optical and X-ray Imaging was used to perform live imaging of tumors developed in Raji-Luc Disseminated model implanted with metastatic A549-LuC cells.



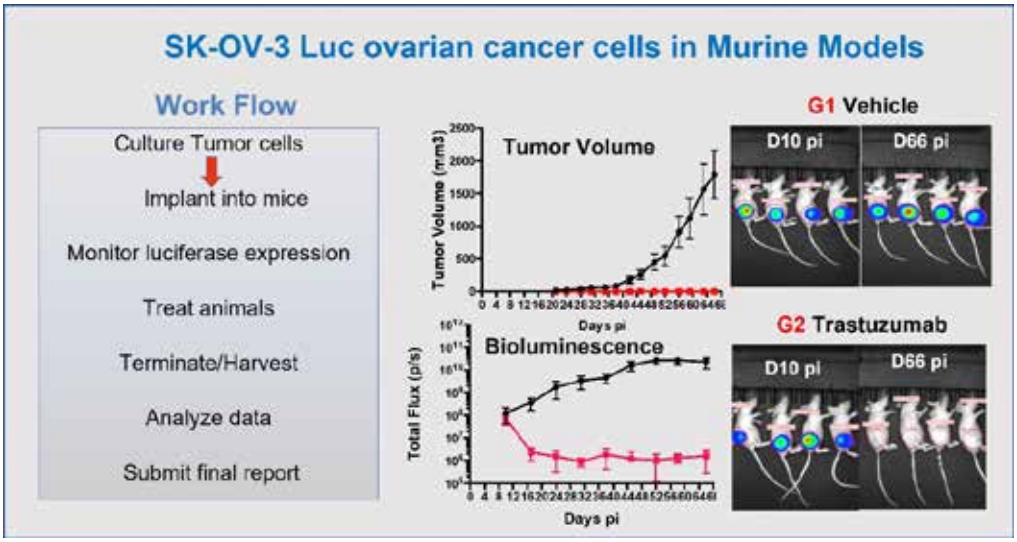
The Luciferase signals captured intensified over 6-18 days of tumor implantation, whereas the signals were weak in treated mice. Similarly, luciferase signal was captured in lungs in intraperitoneal or sub-cutaneous tumors (SKOV-3 xenograft model) and IntegriSenseTM 645 signal in BX PC3-Luc orthotopic model.

Live Imaging of SKOV-3 xenograft with IntegriSenseTM 645



Case study 6: *In-Vivo* Bioluminescent Imaging

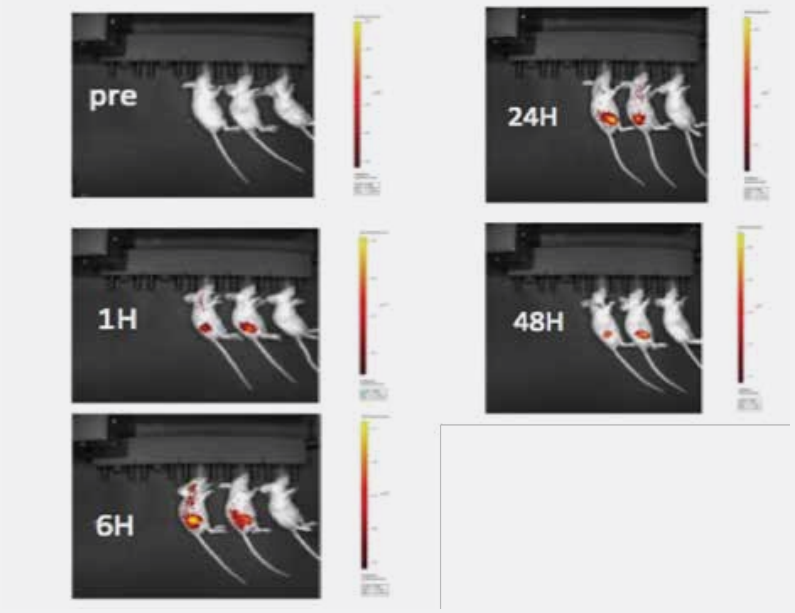
This study in murine tumor shows the early detection of SK-OV-3 luc ovarian cancer cells implanted intraperitoneally or sub-cutaneously regions even before they form clearly visible tumors. Such a study might give a tumor development timeline that may be used to develop a window for treatment with anti-cancer therapeutics. The graphs show the increase in tumor volume over time and the treatment with Trastuzumab reduced the tumor growth. Luciferase imaging intensity was high in untreated mice while the intensity decreased in treated mice, suggesting the anti-tumor effect of Trastuzumab. Also shown is the workflow for this study developed in consultation with the clients or partners.



Case study 7: *In-Vivo* Fluorescent Images of SKOV-3 xenograft using IntegriSense TM 645.

IntegriSense is a targeted fluorescent imaging agent comprising a potent, selective non-peptide small molecule integrin avb3 antagonist tagged with NIR fluorochrome. It was developed to enable *in vivo* visualization and quantification of integrin expressed in tumor cells as well as in neo vasculature, and monitor tumor growth, tumor angiogenesis and treatment efficacy. This study in murine xenograft model shows the images of intraperitoneal SK-OV-3 tumor at 1-48 hrs. Post-IntegriSense TM 645 administration (100 mL/mouse) via tail vein to 2 mice (LEFT) and vehicle administered to 1 mouse (RIGHT).

Note that the tumor is undetectable in pre-IntegriSense TM 645 administered mice.



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Let's begin the Conversation

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