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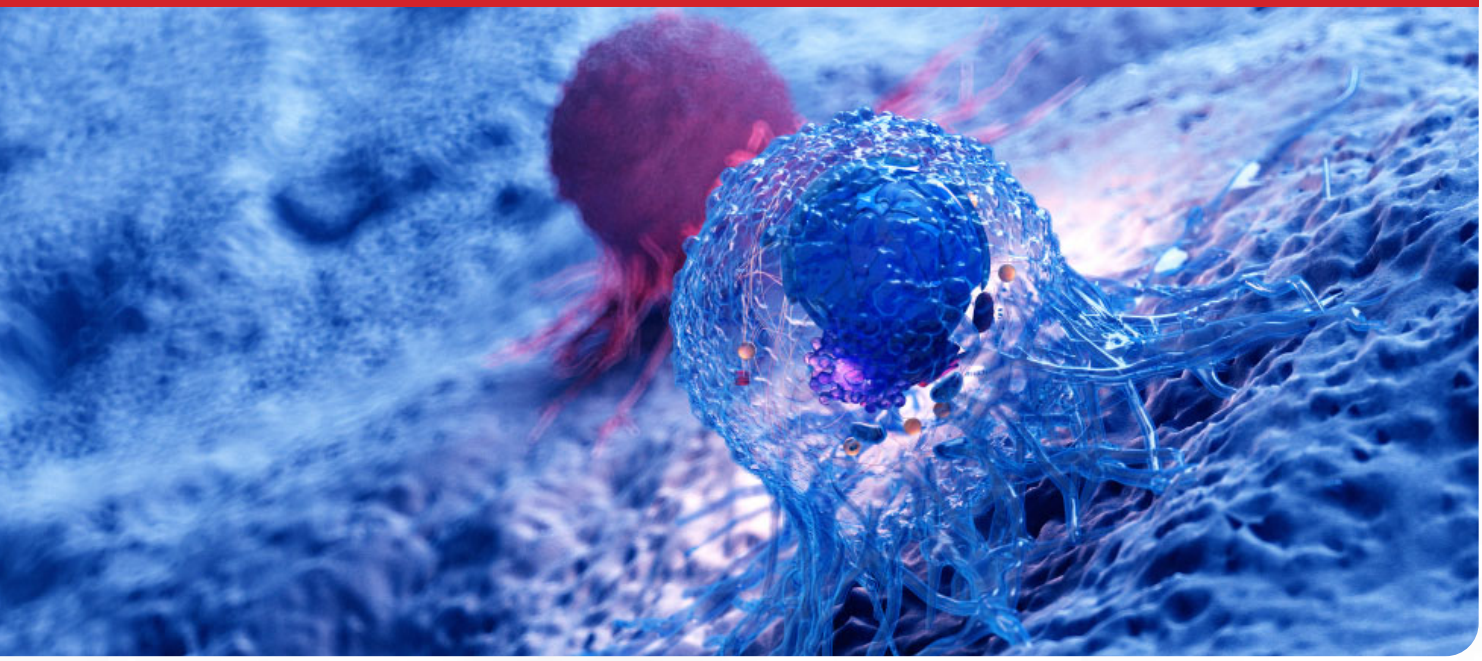
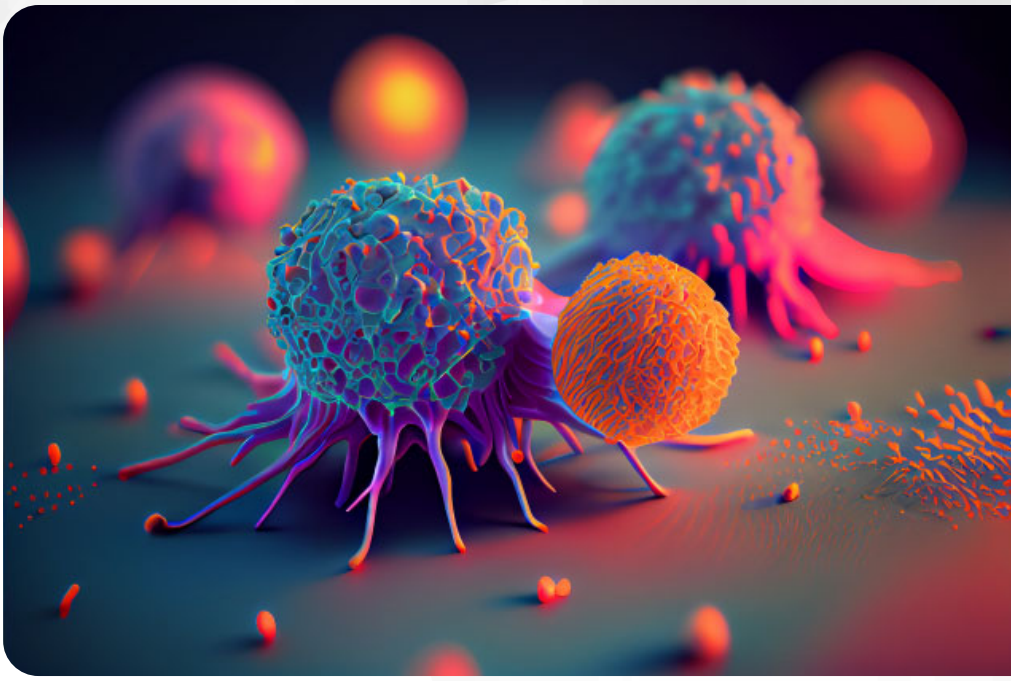
THE DEVELOPMENT JOURNEY OF AN ANTICANCER THERAPY FROM TARGET IDENTIFICATION TO IND-ENABLING STUDIES



INTRODUCTION

Cancer continues to be the second most common disease group in the United States behind heart disease. The American Cancer Society estimates that close to 2 million new cancer cases and over 600,000 deaths from cancer are expected to occur in the US in 2022[1]. However, the death rate due to cancer has been steadily increasing in the past three decades[1], likely due to a combination of improved lifestyle choices, early diagnoses and improved targeted therapies. Traditional therapeutic approaches have included surgery to excise accessible tumors and relatively nonspecific chemotherapies and radiation therapies. These approaches have had limited success but more recently, several therapies have been commercialized that have been successful – the most notable success has been pembrolizumab (Keytruda®). The 2022 approvals included 28 new drug approvals and 2 accelerated to full approvals[2]. The range of modalities that were approved by the FDA is broad and includes multiple bispecific antibodies, a radiotherapeutic, a CAR T-cell therapy and an antibody drug conjugate (ADC) as well as targeted small molecules[2]. Due to the clinical success and commercial adoption of new modalities, there is active drug development across various modalities including small molecules, monoclonal antibodies (mAbs) including bispecifics, ADCs, cell and gene therapies. The preclinical drug development process for the different modalities typically follows an established workflow that covers target identification, hit finding, lead candidate optimization and preclinical efficacy and safety testing. However, it is important to note that the study designs and candidate selection process vary significantly between modalities.



Small molecule candidates are typically identified via screening of large compound libraries using standard high throughput assays. Increasingly, artificial intelligence (AI) methods are being used for smarter drug design where algorithms are used to identify desired components of a chemical structure. Additionally, AI has the capability to process large amounts of data very rapidly resulting in faster identification of small molecule candidates. Monoclonal antibodies can be identified and developed using several methods – the traditional method has been hybridoma based where B cells from mice immunized with the target protein of interest are fused with myeloma cells to generate immortal antibody producing cells. The resulting antibodies are then humanized or engineered to form chimeric antibodies with human reactivity[3]. Methods such as phage display or transgenic mice are preferred as they generate fully human antibodies[3]. Another method is direct screening of B cells expressing the antibodies of interest to identify the relevant heavy and light chain sequences that are then cloned and expressed as recombinant human monoclonal antibodies[3]. Bispecific antibodies, by definition, bind to two different targets, and are often used to bring T-cells closer to the tumor cells to effect killing. The commercially available bispecific antibody therapies have been generated using an innovative approach called "knobs-in-holes" that was developed at Genentech[4]. This method allows the dimerization of two vertical halves of the Y-shaped antibody in a precise way to allow binding of two different targets by the same antibody[4]. As bispecific antibodies are showing increased success in the clinic, there is active research ongoing to improve the development process. Cell therapies targeting cancer typically harness the abilities of immune cells to attack and kill tumor cells. The most well-known cancer cell therapies are CAR T-cells that express chimeric antigen receptors or CARs to target T-cells to tumor cells. As of 2023, 6 CAR T-cell therapies have been commercially approved[5] and all are autologous where the cancer patient's immune cells are isolated, engineered to express the CAR and then re-introduced to the patient. Autologous cell therapies have shown clinical success but the manufacturing process is not scalable so the costs are extremely high. Additionally, the approved autologous cell therapies target hematologic malignancies such as lymphomas and multiple myelomas[5], so targeting solid tumors remains a challenge. Allogeneic cancer cell therapies are developed from healthy donors and can be manufactured at scale but the major challenges are T-cell rejection and graft vs host disease where the patient rejects the engineered T-cells. However, drug developers are using sophisticated genetic engineering approaches to deplete specific proteins that cause immune

rejection and a report in February 2023 demonstrated that an allogeneic cancer cell therapy showed some efficacy with acceptable toxicity in multiple myeloma patients[6].

Independent of the modality type, all candidate therapies are evaluated for preclinical efficacy, safety, ADME and biodistribution. The following sections highlight some of the assays and studies used to evaluate different modalities including small molecules, monoclonal antibodies and cell therapies.

IN VITRO ASSAYS TO IDENTIFY, SCREEN AND EVALUATE NOVEL ANTI-CANCER THERAPIES

The simplest *in vitro* assays to study anticancer therapies are 2D commercially available cell lines that are cultured quickly and economically, and can be used to screen compounds or modalities with anti-cancer activities and evaluate signaling changes in the presence of oncogenes or tumor suppressors. Increasingly, patient derived cells and organoids are increasingly being used as more translational *in vitro* models. Oncology cell-based models are easier to establish than other disease areas as tumors can grow in optimized culture conditions and can retain most of the original tumor hallmarks. The most commonly used endpoint to evaluate anticancer therapies is tumor cell killing which is typically measured using apoptotic markers like cleaved caspase-3 and reduction in cell proliferation.

Target engagement by the therapy can be determined in cell-based models using established readouts – for example, if a drug is a targeted kinase inhibitor, a cell-based model can be used to determine changes in target phosphorylation and downstream signaling in the presence or absence of drug. Signaling changes in the presence of drug can be measured at the transcript level (RNA) or protein level that includes changes in post-translational modification. Transcriptomics analysis is most commonly performed via RNA-Sequencing which is a superior method to the traditional microarray method[7]. RNA-Sequencing gives a global view of changes in gene expression upon drug perturbation and is a good way to identify unexpected expression changes or off-target effects. Similarly, proteomics methods that are typically performed by mass-spectrometry methods result in a global view of protein expression. Along with the global transcriptomics approach, changes in specific mRNA levels can be detected using PCR based methods and the most commonly used method is quantitative RT-PCR (q RT-PCR) which uses synthetic primers to amplify specific transcripts[8]. Microarray hybridization is a good method to get a snapshot of changes in known transcripts[8]. Changes in protein level can be measured by multiple methods including Western blotting, plate-based ELISA assays, bead-based assays such as Luminex®, immunocytochemistry or flow cytometry. Protein assays can be broadly segmented into lysate based and localization-based assays – Western blotting and ELISA based methods measure changes in protein level in a whole cell lysate while immunocytochemistry or immunofluorescence (IF) and flow cytometry are used to measure changes in protein expression and localization. IF allows the visualization of protein expression in specific organelles or cellular regions and employs software to quantitatively measure changes in expression levels and movement of protein within the cell. Flow cytometry analyzes single cells as they travel past lasers in a cytometer and is amenable to complex multiplexing to measure changes in cell surface markers as well as intracellular proteins. As the oncology drug development field has focused increasingly on immune-oncology, *in vitro* assays that measure activation of the immune response such as cytokine expression levels and histological analysis of tumor cell infiltration are used to assess the efficacy of immune-oncology therapies.

While there are numerous assays available to study mechanism of action of specific drugs and identify biomarkers, it is important to note that cell-based models have limited applications and are not truly representative of an *in vivo* tumor.

ANIMAL MODELS TO EVALUATE PRECLINICAL EFFICACY AND PHARMACOLOGY OF NOVEL ANTICANCER THERAPIES

Animal models have long been the gold standard to evaluate efficacy, safety and biodistribution of anticancer therapies. While there have been a few large animal models, mouse models have been the mainstay of cancer drug development. The earliest mouse models were developed using chemical carcinogens to induce tumor formation but in the past few decades, the most commonly used models are either cell-derived or patient-derived xenograft (CDX/PDX) models and genetically engineered mouse models (GEMMs)[9]. CDX models are relatively simple to develop as cancer cell lines are implanted into immune-deficient mice to form tumors. However, CDX models typically do not recapitulate tumor architecture or morphology and are less translational. In contrast, PDX models are developed by the implantation of patient tumor tissues that are isolated via biopsy or surgical excision. The implanted tumor contains the original tumor microenvironment and heterogeneous tumor architecture so PDX models have translational value to evaluate anticancer therapies. However, CDX and PDX models implanted in immune-deficient mice cannot be used to evaluate checkpoint inhibitors or other immune-oncology therapies. The development of humanized mouse models suitable for PDX implantation have been demonstrated to be good models to evaluate immune-oncology therapies such as nivolumab. Currently, humanized mouse PDX models are autologous where the implanted PDX and immune cells are from the same patient. The first model reported in 2022 combined metastatic clear cell renal cell carcinoma PDX with CD34+ bone marrow cells from the same patient[10]. Another report combined colorectal cancer xenografts with human PBMCs in immune-compromised NSG mice to evaluate a combination therapy of nivolumab (anti-PD1 therapy) and regorafenib (a multi-kinase inhibitor)[11]. Syngeneic models are another transplant-based model where mice with an intact immune system are injected with mouse tumor cells derived from mice with the same genetic background. Essentially, syngeneic models are mouse focused where a mouse tumor is evaluated in the context of a mouse immune system. Syngeneic models are reliable and cost-effective and can be used for short-lived efficacy studies. However, there are limited number of syngeneic cell lines and models and in many cases, limited translation to human disease.

GEMMs have been developed for decades and are powered by active research across multiple tumor types to identify and characterize oncogenes, tumor suppressors and aberrant signaling changes that cause tumor development and metastasis. GEMMs have broad applications across the drug development continuum from basic tumor biology studies, to evaluating efficacy and safety of novel therapies and identification of prognostic biomarkers[12]. Robust and efficient gene editing methods such as CRISPR-Cas, Cre-loxP, RNA interference and other have revolutionized the development of GEMMs especially as these methods are becoming more precise with less off-target effects[13]. Developing GEMMs is an expensive and time-consuming exercise but if a GEMM is successfully developed, it can be used to study disease development and progression, identify biomarkers and is a robust model to evaluate the efficacy and safety of novel therapies.

Since there are thousands of mouse models of cancer available for different tumor types, it is essential to utilize tumor biology and cell-based assay data to identify the optimal *in vivo* model for each study to ensure that physiologically relevant data are generated to move a therapy forward into ADME and safety studies.

BIODISTRIBUTION AND PK/PD STUDIES FOR ANTICANCER THERAPIES

Bioanalytical studies can be segmented as follows: pharmacokinetics (PK), toxicokinetics (TK), pharmacodynamics (PD), immunogenicity assays and biomarker studies. Pharmacokinetic studies give information on the duration and intensity of a therapeutic response, where the drug candidate introduced into an animal model and the amount of drug in the serum or plasma is assessed to identify the bioavailability and clearance of the drug. Toxicokinetic studies are similar to PK studies except the drug dosage is significantly higher and the study objective is to determine acceptable drug exposure levels. Pharmacodynamic studies ascertain the effect of the drug on the animal model, and identify target binding and impact on downstream signaling or gene expression. Several anticancer therapies trigger an immune response, so immunogenicity assays that measure changes in the immune system or assess the neutralizing antibody responses are critical readouts.

Biomarkers are critical endpoints to measure drug efficacy in preclinical and clinical models, so it is essential to validate identified biomarkers in preclinical models. Biomarker validation is considered to be a part of bioanalysis, and the same standards including specificity, sensitivity, robustness and reproducibility are applied. The FDA's bioanalytical method validation guidelines also highlights the requirement for validation biomarkers using bioanalysis standards[14]. The most common methods used in bioanalysis include cell-based assays, ligand binding assays, mass spectrometry-based methods, flow cytometry and more recently, single molecule analysis. *In vitro* binding and potency assays are commonly used to assess drugs prior to more detailed ADME studies in animal models. Cell-based assays are used to assess biological activity and the assay of choice depends on the drug modality. For example, the potency of therapeutic monoclonal antibodies is typically assessed using binding assays. Cell-based assay readouts include cell proliferation or tumor killing assays or changes in downstream signaling that drive cell proliferation and metastasis.

Ligand binding assays (LBAs) cover a broad range of assays from radioimmunoassay (RIA) to ELISA to high throughput assays such as MSD. These assays use the sandwich format where an immobilized capture antibody binds the analyte of interest that is then detected using another antibody, which is typically conjugated to a readout for detection. Mass spectrometry (MS) methods are widely used in bioanalysis and has distinct advantages over LBAs. MS methods are more selective, amenable to multiplexing and can simultaneously identify and quantify both total drug and metabolites. Mass spectrometry is combined with either liquid or gas chromatography (LC-MS or GC-MS). LC-MS is widely used to analyze large molecule therapeutics including mAbs and antibody-drug conjugates (ADCs)[15]. LC-MS can be combined with LBAs either as complementary assays or orthogonal assays where the LBA data is confirmed independently by LC-MS analysis[16].

Flow cytometry has been the gold standard in many areas including immune cell profiling and cell signaling studies. The technology measures the properties of single cells and provides multiplexed data on different cell populations. Flow cytometry is increasingly being used in the bioanalysis of large molecule drugs especially for cancer cell therapies. Whole cell therapies such as CAR-T cells can be comprehensively assessed using relevant flow cytometry panels[17]. Additionally, flow cytometry is also used to detect the presence of anti-drug antibodies that can inhibit the function of the drug. Single molecule analysis assays are an emerging tool in the bioanalysis toolbox. These assays offer extremely high levels of sensitivity molecules and are also amenable to automation to improve throughput. Some of the better-known platforms are the Simoa from Quanterix and SMC Erenna from EMD Millipore. Simoa (single molecule array) used bead conjugated antibodies to capture low abundance protein[18] while the Erenna platform uses antibodies to capture analytes that are detected using fluorescently conjugated detection antibodies.

SECTION 4: PRECLINICAL TOXICOLOGY STUDIES

Once a drug's efficacy is known, it is essential to understand dosing ranges and toxicities at specific doses[19]. The introduction of the ICH S9 guidance from the FDA was originally published in 2010 and initially outlined the safety requirements for anticancer therapies. Since then, there have been follow on documents that share more information on the regulatory guidelines for anticancer therapies[20]. The ICH S9 guidelines have helped streamline the safety requirements that are different from therapies targeting other diseases. For example, abuse liability testing and reproductive toxicity testing are not necessarily required[21]. Additionally, the required safety studies vary depending on the modality under investigation. For small molecule anticancer therapies, safety studies begin with dose-ranging studies in one small animal and one large animal species. Genetic toxicology is typically tested using the bacterial Ames test which has been a well-established assay to evaluate therapies that cause DNA damage and mutations. If the Ames test is negative but other chromosomal abnormalities are detected, then additional testing may be required. Safety pharmacology studies are an essential component of preclinical toxicology studies and evaluate the pharmacodynamic of the anticancer therapy or combination therapies that are being investigated. These studies include several tests to evaluate the effect of the therapy on vital functions such as cardiovascular, lung and CNS functions[22]. The hERG test is a well-known test to assess cardiovascular liability while lung and CNS safety studies are typically performed in rodent models.

Monoclonal antibody-based therapies also require immunogenicity testing, which identifies if a monoclonal antibody-based therapy triggers the formation of anti-drug antibodies or ADAs. Immunogenicity testing is typically performed early in the safety testing process to ensure that monoclonal antibodies and derivatives such as antibody-drug conjugates can be administered without triggering a massive immune response. It is important to note that genetic toxicology and safety pharmacology studies are performed for mAb-based therapies as well. Complex therapies such as cell and gene therapies require unique safety study design, as the studies should ideally mimic the clinical trial plan in terms of dosing, route of administration, clearance of viral vectors or engineered cells etc. In some cases, the optimal approach may be to combine pharmacology and safety in hybrid studies in *in vitro* and *in vivo* models. Unlike small molecules or mAb therapies, the regulatory guidelines for cell and gene therapy safety studies are still

evolving and the FDA publishes multiple guidance documents to support IND-enabling studies for various cell and gene therapies[23]. Therefore, it is important that drug developers work closely with regulatory agencies to ensure that preclinical safety studies meet the requirements for toxicity and tolerability.

Given the complexity of developing new anticancer therapies or novel combination therapies, it is no surprise that the development process for an anti-cancer therapy can take 6-12 years[24] and cost anywhere between $944 million and $4.5 billion[25]. This process cannot be a sole endeavor for a biopharma company and increasingly, they are partnering with CROs and CDMOs to expedite specific stages of the drug development process.

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