Measuring Cellular Kinetics

Subjects: Oncology

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Novel modalities such as cell and gene therapies have proven to be efficacious for numerous clinical indications–primarily in rare disease and immune oncology. Because of this success, drug developers are heavily investing in these novel modalities. Given the complexity of these therapeutics, a variety of bioanalytical techniques are employed to fully characterize the pharmacokinetics of these therapies in clinical studies. Industry trends indicate that quantitative PCR (qPCR) and multiparameter flow cytometry are both valuable in determining the pharmacokinetics, i.e. cellular kinetics, of cell therapies.

pharmacokinetics PK cellular kinetics

1. Introduction

Drug development has been evolving dramatically over the past several decades to counter the unmet challenge of previously untreatable diseases. Novel and refined technologies have propelled drug developers beyond small molecule therapeutics and simple biologics (e.g., monoclonal antibodies) into highly complex, large-molecule drugs. Of these complex modalities, the class of cell and gene therapies has progressed exponentially over the last several years, resulting in successful regulatory approvals of several cell and gene therapies for various treatment indications. One such indication is immune oncology; the interplay of the immune system and diseases such as cancer or autoimmunity has led to many breakthroughs in immunotherapy. Additionally, advancements in gene editing techniques and transgene delivery systems are leading to breakthroughs in patient care. Various gene and cell therapy programs have shown great promise for treatment of a multitude of diseases including those in rare disease and difficult to treat cancers.

Gene therapy is the introduction of genetic material into a patient for treatment of a disease. The transfer of genetic material or method of delivery is commonly achieved via viral (e.g., adenovirus, lentivirus, AAV (adeno-associated virus) and non-viral vectors (e.g., lipid nanoparticles, liposomes, polymers, and peptides). Regardless of delivery method, gene therapies can be classified as in vivo or ex vivo. In vivo gene therapies are administered directly to the patient. whereas ex vivo gene therapies entail harvesting patient's cells for genetic modification outside of the patient with a therapeutic transgene, and then returning the cells to the patient [1][2]. Ex vivo gene therapy can be further classified as adoptive cell therapies that are differentiated into subclasses based on their mechanism of action. Of these, tumor-infiltrating lymphocytes (TILs), T-cell receptor (TCR) therapies, and chimeric antigen receptor (CAR) modified immune cells are of current interest for drug developers.

Although extremely attractive and potentially opportunistic, the development of CAR-based therapeutics is not without challenges that have been summarized in several recent reviews [3][4][5]. An additional degree of complexity of CAR-based therapeutics is the source of cells being delivered to the patient. The cells can be autologous, i.e., derived from the patient's own cells, or allogeneic, wherein the cells are derived from a source other than the patient. While autologous cell therapies are considered to be safer, significant time in the preparation of these modalities can lead to complications for the sick patient. In contrast, allogeneic cell therapies can be readily available, often referred to as "off-the-shelf" therapeutic products. While allogeneic sources have great clinical potential, significant concerns remain. Namely, rejection in the form of graft versus host disease (GvHD) or other unwanted immune reactions to foreign cells threatens patient safety and cell therapy efficacy [6].

Despite challenges, these therapies have shown remarkable effectiveness in treating different types of blood cancers and have led to the FDA (Food and Drug Administration) approval of several cell therapies to-date, including Kymriah® (tisagenlecleucel) for acute lymphoblastic leukemia [7], Tecartus™ (brexucabtagene autoleucel) for mantle cell lymphoma, Yescarta® (axicabtagene ciloleucel) for B-cell lymphoma and Bryanzi (lisocabtagene maraleucel) for relapsed and refractory large B-cell lymphomas, with all four therapies being autologous CAR-T cells that target CD19 on the surface of B cells [8][9]. The most recent cell therapy approval was for Carvykt (ciltacabtagene autoleucel), an autologous BCMA-directed CAR-T therapy for the treatment of relapsed and refractory multiple myeloma [10]. The approval of these cell therapies is likely only the beginning. The cell-therapy landscape continues to evolve with refinement of CAR constructs, targeting a variety of disease-related antigens, and harnessing other immune cells to expand into solid tumor oncology and autoimmune disorders [11].

Cell therapies are "living cells." Therefore, they exhibit a unique pharmacokinetic (PK) profile termed cellular kinetics. Cellular kinetic profiles, specifically for CAR-T therapeutics, appear to have multiphasic parameters that include distribution, expansion, contraction, and persistence phases. Moreover, with the characteristic of rapid in vivo expansion, cell therapies can persist up to one year or longer in patients. Therefore, unlike typical small or large molecule therapies, cellular therapies do not show a strong relationship between dose and exposure. Thus, it is imperative to effectively strategize bioanalytical sampling for these studies. The cellular kinetics of the first approved CAR-T cell therapy, Kymriah, were measured and described by Mueller et al. [12], showing the expansion and persistence of the circulating cell therapy in clinical samples.

Cellular kinetics can be quantified using multiple bioanalytical techniques. Although correlative, the different techniques may not always produce concordant data sets. Thus, it is imperative to understand the platform being used, know what is being quantified, and how to compare and interpret the data. Current industry trends utilize two main techniques for resolving cellular kinetics–quantitative polymerase chain reaction (qPCR) and flow cytometry.

With CAR-T cell therapy or other cell therapies, an engineered transgene that encodes the CAR or cell surface therapeutic is introduced in the form of RNA. The RNA is reverse-transcribed into DNA, which is integrated into the genome of the patient's cells to express the CAR protein at the cell surface. qPCR measures cell concentration through detection of integrated transgene copies per cell. These data are then extrapolated or inferred as the number of circulating CAR-T cells. Whereas flow cytometry provides a direct measure of the cell therapy via

antibody-specific fluorescent labeling of the engineered cell surface molecule (e.g., the CAR). The difference in detection of CAR-T therapy between qPCR and flow cytometry is illustrated in **Figure 1**. Both platforms are valuable in measuring cellular kinetics, and both platforms have advantages and disadvantages. qPCR and flow cytometry will be discussed in the context of measuring cellular kinetics, and common considerations when developing and validating these assays will be addressed.

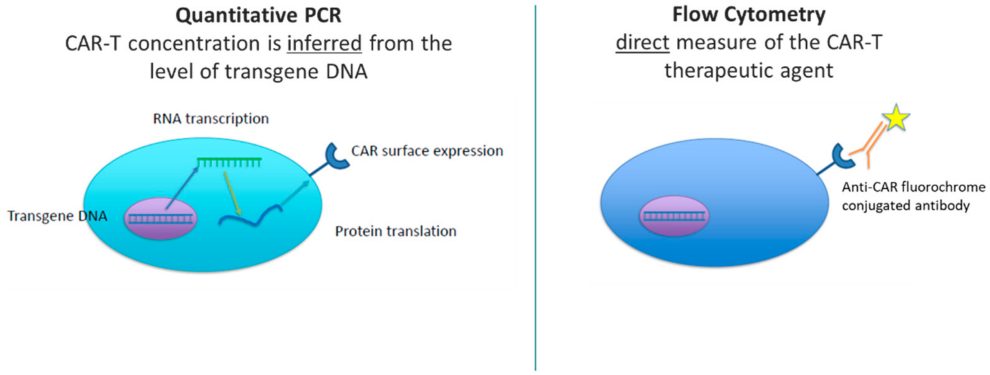

Figure 1. Schematic of CAR-T Detection by qPCR and Flow Cytometry.

2. Quantitative PCR

From its invention in the early 1980s using oligonucleotide primers, dNTPs, and Taq polymerase for DNA amplification [13], the qPCR workflow has evolved and been used in various applications in food safety, agriculture, diagnostics, and drug development. qPCR is a technique used to amplify and quantify DNA. This versatile platform has many advantages. qPCR assays are rapid, highly specific, robust, and provide exceptional target sensitivity. qPCR remains the 'gold standard' for nucleic acid quantification. Moreover, for cellular kinetics, qPCR is the standard for measuring transgene DNA. Reverse transcription qPCR (RT-qPCR) entails transcribing RNA into complementary DNA (cDNA) and using the cDNA as a template for the PCR reaction. RT-qPCR applications are not typically employed in cellular kinetics and are out of scope for this manuscript.

Another molecular platform undergoing significant adoption is digital PCR (dPCR) technology. The concept of dPCR was first described by Sykes et al. wherein the absolute quantification of nucleic acids was achievable through end-point PCR and Poisson statistics [14]. dPCR's hallmark capability is absolute quantification of nucleic acid concentration in a sample, thereby eliminating the need for a standard curve. The dPCR workflow is summarized in the following sequential steps: sample preparation, partitioning, amplification, and detection. Numerous commercial dPCR platforms have been developed over the years utilizing differing partitioning techniques, though droplet digital PCR (ddPCR) technology is widely used in cell therapy bioanalysis. For dPCR, the sample is prepared just as it would be prepared for traditional qPCR. Then, in the case of ddPCR specifically, the sample is partitioned into uniform droplets that have a few or no target sequences. After the droplets are generated, PCR is used to amplify the sequences. The droplets are then passed through a droplet reader that separates them into positive or negative droplets based on the amount of fluorescence that is above or below a threshold; hence the 'digital' aspect; droplets are either fluorescent or they are not. Finally, to calculate the

concentration of target in the sample, the technology uses the ratio of positive to total droplets and Poisson distribution statistics [15]. In recent industry trends, both qPCR and various dPCR platforms have been heavily utilized in determining cellular kinetics to determine the number of cells that make up the cellular therapy pharmacokinetic profile. Previous efforts have compared both platforms and advantages of each [16]. One of the most cited advantages of using dPCR over qPCR is the mere fact that a standard curve is not required for absolute quantification of copy numbers. Another attractive reason for developing a dPCR assay is the minimal susceptibility to matrix interferents and PCR inhibitors. Both qPCR and dPCR have comparable sensitivity capabilities. However, an advantage of the dPCR system is superior precision for rare events and at lower copy numbers. Therefore, dPCR has been slightly preferred for the reason of requiring adequate precision at lower sensitivity to detect as low a number of cells as possible for characterizing persistence of cell therapy products. Another advantage of qPCR is the high-throughput capability of the platform, especially with 384-well plate formats. This advantage lends favor for deploying this platform for assays that are used to support global or multi-site clinical trials. Lastly, the cost of qPCR reagents and consumables are more affordable than the often proprietary reagents for dPCR platforms.

Method Considerations

Collectively, whether using qPCR or dPCR, molecular assays are one of the primary methods for measuring cellular kinetics. To that end, these assays measure the concentration of the therapeutic from the level of transgene DNA in matrix (e.g., peripheral blood, bone marrow, etc.). With cellular kinetics, the primary advantage of qPCR assays over flow cytometry is superior sensitivity. Although not a direct measure of the CAR, qPCR remains the more sensitive method [17] and is reported as transgene copies/µg of DNA or vector copies/mL of blood.

Considerations for developing and validating these assays have been extensively described in recent publications. In general, a qPCR/dPCR method is developed for assessing two targets; the first is designed to detect the CAR transgene DNA and a second that measures a reference gene DNA used to normalize the transgene copy number within each sample. Vector copy number (VCN) assessment measures presence of the CAR vector and CAR cells by determining the average vector copies per genome by using the CAR copy number and genome copies together. In their publication, Yang and Doddareddy [18] summarize recommendations and best practices for developing cellular kinetics assays for CAR therapies via qPCR. Notably, reliability of the VCN can be highly dependent on the specificity of the primers and probes to the target [19], as the technology can slightly overestimate the number of CAR cells in a sample. Universal qPCR assays to detect CAR-T cells in peripheral blood have been described [20]. Such methods can be employed to monitor cellular kinetics for CAR based therapies.

Like all bioanalytical assays, the quality of the sample preparation dictates the quality of results. When developing qPCR for cellular kinetics assays, the nucleic acid extraction method is critical. Different DNA extraction methods (e.g., silica-based versus magnetic bead-based, manual versus automated extraction) can result in varying nucleic acid yields. Thus, the DNA extraction method requires optimization to yield sufficient nucleic acid recovery and highest purity. Another prominently debated topic concerns the use of dPCR and determining the “nominal” concentration of reference material. While the use of absolute quantification is advantageous to avoid the requirement of preparing a standard curve containing known spike concentrations of a reference calibrator,

challenges arise in determining which method of concentration determination will provide the most accurate value of the starting calibrator material. For example, when nucleic acid concentration is determined by a default method such as UV absorption, an overestimation of the actual concentration may occur due to the presence of contaminants such as salts, organic solvents, or detergents. Another drawback of using dPCR for cellular kinetics assays is the limited dynamic range. The upper limit of detection is limited by the saturation of positive droplets [21]. Therefore, when using a reference gene, the maximum amount of genomic DNA (gDNA) input in the reaction must be considered to ensure reference gene copies are within the dynamic range of detection.

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