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Research paper

Implementation of design of experiments (DOE) in the development and validation of a cell-based bioassay for the detection of anti-drug neutralizing antibodies in human serum

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ABSTRACT

The administration of biological therapeutics can potentially elicit the development of neutralizing antibodies (NAbs) to the therapeutic drug in patients, which could have a significant impact on drug efficacy and safety. A rigorous *in vitro* cell-based assay for the detection of NAbs is critical for the assessment of the immunogenicity profile of the therapeutic drug. Conatumumab is a fully human monoclonal agonist antibody directed against the extracellular domain of human TRAIL receptor 2 (TR-2). It is being investigated as a cancer treatment because it is able to induce apoptosis in sensitive tumor cells. This report demonstrates how statistically designed experiments could be employed effectively in different stages of a NAb bioassay life cycle in order to characterize, optimize and stabilize the assay with added benefit of resource efficiency. By combining the approach of design of experiments (DOE) with subject matter expertise and experience, we were able to understand thoroughly how assay parameters affect the performance of the assay individually and interactively, identify the key assay parameters, define assay operating ranges and finally achieve a robust and sensitive cell-based assay for the detection of NAbs to Conatumumab. With the goal of developing a cell-based bioassay that is highly optimized for sensitivity, specificity, precision, and robustness, we performed 2 DOE experiments for assay optimization and 1 DOE experiment to validate assay robustness. We evaluated key operating parameters of the assay such as cell number, percentage of serum matrix, concentration of the therapeutic drug, concentration of the cross-linker, length of various incubation steps, cell age, interval between cell subculture and bioassay time, and detection equipment.

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1. Introduction

Biological therapeutics can potentially elicit immune responses in animal and human subjects receiving the treatment, therefore impacting drug efficacy and safety (Shankar et al., 2008). It is essential that well-designed and developed assays are used to evaluate the immune responses associated with the treatment of the drug. When neutralizing antibodies (NAbs) are developed against the drug, the biological activity of the therapeutic molecule is diminished. Cell-based assays, which resemble closely the mechanism of action of the therapeutic molecule *in vivo*, can be used for the assessment of anti-drug product NAbs (Gupta et al., 2007). Such assays can aid the evaluation of the immunogenicity of the molecule.

Based on the knowledge accumulated from early discovery experimentation, important components for a well-designed cell-based NAb assay can include but are not limited to the type of assay format, the type of assay endpoint,

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the type of cell line, the number of cells, the concentration of the therapeutic drug, the concentration of the ligand, the percentage of serum matrix, the incubation time, the desirable assay sensitivity, *etc*. More knowledge of these key assay parameters need to be generated through additional experimentation. While the traditional one-factor-at-a-time approach is still useful in certain situations, the implementation of experimental design with statistical considerations more often than not yields data that can be analyzed to serve the research purpose more efficiently and effectively, ultimately improving assay quality while shortening the development time (Antony 2003). It allows for systematic, structured and organized evaluation of the assay parameters and their interactions for a variety of purposes depending on the stage of the assay life cycle (Altan et al., 2010). For example, in assay development, the purpose of implementing design of experiments (DOE) can be characterization, screening, optimization and robustness evaluation *etc*.; in assay validation, DOE can be used for confirmation and for studying assay robustness (Dejaegher and Heyden, 2007). In this manuscript, we report the successful implementation of this dynamic scientific information gathering approach in the development and validation of a robust and sensitive cell-based bioassay for the detection of NAbs to Conatumumab, a potential anti-cancer therapy currently under development at Amgen, Inc.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) binds to five tumor necrosis factor (TNF) receptor superfamily members. Binding of TRAIL to TRAIL receptor 1 (TR 1) and TRAIL receptor 2 (TR2) leads to programmed cell death or apoptosis (Rowinsky 2005). TR-2 is widely expressed on transformed human cells and most primary human tumor tissues. Targeting the TRAIL receptors represents a therapeutic strategy to destroy cancer cells via targeted induction of apoptosis, a complex cell signaling cascade that is tightly regulated (Duiker et al., 2006). Conatumumab is a fully human monoclonal agonist antibody directed against the extracellular domain of human TR-2. Caspases, a family of cysteine proteases, play essential roles in apoptosis. Caspases are first synthesized as inactive zymogens in cells and are cleaved before forming active heterotetramers that drive apoptotic events. Therefore, cellular apoptosis can be monitored through the measurement of caspase activation (Elmore 2007). It has been shown that Conatumumab activates intracellular caspases *in vitro* in the presence of a cross-linker such as protein G. The function of such cross-linker is thought to oligomerize the TRAIL receptors on cells. Furthermore, it has been demonstrated that Conatumumab inhibits tumor growth in colon, lung, and pancreatic xenograft models. It has been hypothesized that the crosslinking function *in vivo* is accomplished by Fc receptor-bearing cells (Kaplan-Lefko et al., 2010).

The cell-based bioassay for the detection of NAbs to Conatumumab in human serum samples described in this manuscript measures the activation of caspase-3/7 enzyme in cells expressing the TR-2 receptor. In general, cell-based bioassays are more variable and have a limited dynamic range when compared with non cell-based competitive ligand-binding assays. In addition, cell-based bioassays are more laborious, therefore they can be more time-consuming for assay development and validation. However, since cell-based bioassays are more reflective of the *in vivo* situation, FDA's guidance on assay development for immunogenicity testing of therapeutic proteins recommends the use of cell-based bioassays whenever it is possible (U.S. Department of Health and Human Services, Food and Drug Administration 2009). DOE is a powerful and efficient tool which was applied successfully to evaluate assay sensitivity and robustness in the development and validation of a cell-based assay for the detection of NAbs to Conatumumab. It was used to assess key operating parameters of the assay such as cell number, percentage of serum matrix, concentration of the therapeutic drug, concentration of the cross-linker, length of various incubation steps, cell age, interval between cell subculture and bioassay time, and detection equipment.

2. Material and methods

2.1. Reagents

COLO 205, DLD-1, HCT-15, H460, and H2122 cell lines were obtained from ATCC (Manassas, VA). RPMI 1640 with L-glutamine, FBS, penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA). Conatumumab and rabbit anti-Conatumumab polyclonal antibodies were provided by Amgen Inc. (Thousand Oaks, CA). Recombinant human TRAIL (rhTRAIL) was obtained from R&D Systems (Minneapolis, MN). Pooled normal human serum (PHS) and individual human serum samples were obtained from Bioreclamation (Hicksville, NY). Recombinant protein G was obtained from Thermo Fisher Scientific Inc (Rockford, IL). Caspase-Glo™ 3/7 assay kit was obtained from Promega (Madison, WI). Assays to measure the caspase-3/7 enzyme activities were performed using the black with clear bottom Costar 96-well tissue culture treated plates.

2.2. Cell culture

COLO 205, DLD-1 and HCT-15 are all human colorectal adenocarcinoma cell lines. H460 is a human large cell lung cancer cell line, while H2122 is a human non-small cell lung cancer cell line. All of them were maintained in RPMI 1640 with L-glutamine, 10% FBS, and 1% penicillin/streptomycin in 37 °C, 5% CO_2, and 90% humidity in Corning/Costar tissue culture treated flasks. The cells were subcultured according to ATCC's recommendations.

2.3. Identification of cell lines responsive to Conatumumab

Five cancer cell lines including COLO205, DLD-1, HCT-15, H460, and H2122 were treated with Conatumumab at different concentrations in the presence of 2.5% pooled human serum matrix for 4 h followed by 2 h incubation with 50 μg/mL of protein G. Conatumumab induced caspase-3/7 enzyme activities were measured using the Caspase-Glo™ 3/7 assay kit according to the manufacturer's recommendations.

2.4. Applying DOE to assay development and validation

In this report, DOE experiments were designed and analyzed using the JMP® statistical software from SAS (Cary, NC). When performing the DOE experiments, reagent

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