Poster # W1230-02-09

In-Vitro Assessment of Comparative Immunogenicity for Generic Synthetic Peptide Drug Products

Eric Pang[1], Kuo-Tang Liao[1], Claire K. Holley[2], Marina A. Dobrovolskaia[2], and Daniela Verthelyi[3]

[1]Office of Research and Standards, Office of Generic Drugs,

[3]Office of Biotechnology Products, Office of Pharmaceutical Quality,
Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, MD, USA

[2]Nanotechnology Characterization Lab, Cancer Research Technology Program, Frederick National Laboratory for Cancer Research Sponsored by the National Cancer Institute, Frederick, MD, USA

CONTACT INFORMATION: Eric Pang, eric.pang@fda.hhs.gov

PURPOSE

To facilitate generic peptide submission and approval, the FDA guidance on *ANDAs for Certain Highly Purified Synthetic Peptide Drug Products That Refer to Listed Drugs of rDNA Origin* (May 2021) provides FDA's recommendations for demonstrating the equivalence of synthetically produced generic peptide drug products that reference a recombinantly produced reference listed drug (RLD). Although the guidance is specific for glucagon, liraglutide, nesiritide, teriparatide, and teduglutide, and FDA has additional PSGs for other peptide products that reference this synthetic peptide guidance. A critical aspect in the development and approval of these peptide products is the need for an immunogenicity risk assessment. This risk is informed by the RLD's clinical experience as well as specific quality aspects of the peptide. If the immunogenicity risk of the RLD peptide is considered to be elevated, additional non-clinical assays (e.g., cell-based or functional binding assays) may be needed for generic applicants to demonstrate that their product will not further elevate the immunogenicity risk. To facilitate the development of these non-clinical immunogenicity assays, we examined commonly used assays for detecting innate immune response mediated impurities (IIRMIs), including cytokine secretion by peripheral blood mononuclear cells (PBMCs). We investigated the formulation (of teriparatide) and storage handling effects on the assay sensitivity and performance.

METHODS

PBMCs were isolated from the whole blood of 10 healthy donor volunteers. To assess various blood storage and handling conditions (summarized in Table 1 below), PBMCs were isolated from fresh whole blood on the same day (fresh PBMC), freshly isolated and incubated for 24 hrs prior to use (cultured PBMC), freshly isolated and cryopreserved before use (cryopreserved PBMC) or isolated from the whole blood stored for either 24 or 48 hours at 4°C (PBMC from 24 hr stored blood and PBMC from 48 hr stored blood, respectively). Teriparatide drug product (FORTEO) and 10 IIRMIs from various sources were tested, each at 4 concentrations. After PBMCs were prepared for culture based on the above conditions, they were incubated with controls, teriparatide drug product, or IIRMIs for 24 hours. Teriparatide drug product and IIRMI controls were tested at various concentrations.

At the end of incubation, the samples were centrifuged for 5 min at 18,000xg, and supernatants were again analyzed for the presence of cytokines using our custom 7-plex from Quansys Biosciences for the detection of IL-1α, IL-6, IL-8, IP-10, MIP-1, MCP-1, and PGE-2.

Table 1: Tested Storage and Handling Conditions for PBMC assay

	Storage/Handling Condition		Experimental Timeline			
			Day 1	Day 2	Day 3	Day 4
1	Fresh Blood	Fresh PBMCs	PBMCs isolated and treated	Supernatants collected		
2		Cultured PBMCs	PBMCs isolated; cultured for 24 hours	PBMCs treated	Supernatants collected	
3		Cryopreserved PBMCs	PBMCs isolated; cryopreserved for 48 hours		PBMCs isolated and treated	Supernatants collected
4		Fresh Whole Blood	whole blood treated	Supernatants collected		
5	24 hour Stored Blood	PBMC/24h stored blood	whole blood refrigerated for 24 hours	PBMCs isolated and treated	Supernatants collected	
6	48 hour Stored Blood	PBMC/48h stored blood	whole blood refrigerated for 48 hours		PBMCs isolated and treated	Supernatants collected

RESULTS

Cytokine Selection

To understand which cytokines to include in our tests, and if we can narrow down to using 3 or 4 out of the 16 cytokines available to us, we tested 10 IIRMI standards individually with all 16 cytokines. A 2-sided paired Wilcoxon test was used to compare cytokines induced by the individual concentration of IIRMI with that in the negative control samples when the data from the negative control were pooled across all donors. A signature cytokine was identified for each IIRMI by determining the lowest IIRMI concentration, which when compared to the baseline, resulted in an elevation of the cytokine and had the lowest p-value (i.e., at least $p<0.05$). If two cytokines reached a level of significance at the same IIRMI concentration, the cytokine with the lower p-value would be considered. Finally, the selected cytokine would be the one with the fewest overlaps between treated samples and controls and was consistent between individual donors.

Using the above method, the top three cytokines for each IIRMI were identified (Table 2). Interestingly, all IIRMIs that activate membrane-tethered toll like receptors (TLRs), consistently induced two cytokines: IL-1 and MIP-1. This finding suggests that any of these two cytokines could be used as a biomarker for the detection of IIRMIs triggering membrane-tethered PRRs. In contrast, no such consistency was observed for IIRMIs that activate endosomal TLRs. Therefore, a combination of cytokines (i.e., MCP-1 and IL-8 or MCP-1 and IL-6) would be helpful to include in order to detect the presence of IIRMIs triggering endosomal TLRs. One of the following cytokines: IL-6, IL-8, or IP-10 could be used to suggest the presence of IIRMIs triggering cytosolic PRRs.

Based on the above finding, we focused on the 7-plex panel which includes a combination of the following signature cytokines: IL-1, MIP-1, IP-10, MCP-1, IL-6 and IL-8. In addition, one cytokine (PGE-2) is used because of its responsiveness to teriparatide.

IIMI	IFNα	IFNγ	IFNλ	IL-10	IL-12	IL-17	IL-1α	IL-1β	IL-2	IL-6	IL-8	IP-10	MCP-1	MIP-1α	TNFα	PGE-2
B. subtilis flagellin	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE	TRUE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE	FALSE	FALSE
FSL-1	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE	TRUE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE	FALSE	FALSE
Zymosan	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE	TRUE	FALSE	FALSE
E. coli O111:B4 LPS	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE	TRUE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE	FALSE	FALSE
ODN 2006	TRUE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE	FALSE	TRUE	FALSE	FALSE	FALSE
Poly(I:C) HMW	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE	FALSE	TRUE	TRUE	FALSE	FALSE	FALSE
Poly(I:C) LMW	FALSE	TRUE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE	TRUE	FALSE	FALSE	FALSE
CLO75	FALSE	FALSE	FALSE	TRUE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE	TRUE	FALSE	FALSE	FALSE	FALSE	FALSE
ODN2216	TRUE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE	TRUE	FALSE	FALSE	FALSE
MDP	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	TRUE	TRUE	TRUE	FALSE	FALSE	FALSE	FALSE

Table 2. Top three signature cytokines induced by individual IIRMI. A 2-sided unpaired Wilcoxon test was used to select the top three cytokines for each IIRMI that had consistent responses between all donors and the lowest p-value. Starting with the lowest concentration for each IIRMI, if three cytokines did not achieve $p \leq 0.05$, the next higher concentration was evaluated until three cytokines were chosen. If more than three cytokines achieved $p \leq 0.05$ at the selected concentration, the three with the lowest p-values were selected. These cytokines are shown in the table as "TRUE". IIRMIs that signal via cellular membrane-tethered pattern recognition receptor (PRRs) are highlighted in blue; those signaling via endosomal PRRs are in red, and cytosolic PRR is in green font.

Key terms:
IL: Interleukin
IFN: Interferon
IP: Interferon gamma inducible protein
MCP: monocyte chemoattractant protein
MIP: Macrophage inflammatory protein
TNF: tumor necrosis factor
PGE-2: Prostaglandin-E_2

Formulation and API Effects

To understand whether the induction of cytokines observed in teriparatide-treated cultures was due to the API peptide or formulation buffer (FB), we tested teriparatide drug (TP) side-by-side with FB at equivalent dilutions (with PBS) that resulted in equivalent concentrations of the FB. To rule out the possibility that lab-made FB may contain IIRMIs which would greatly enhance or stimulate cytokine releases, we also performed TP dilutions in the FB and tested them in the same cultures with PBS-diluted FB and TP. The results of this experiment demonstrated that PGE-2 and IL-8 responds to TP is due to FB (Figure 1).

To narrow down which component in the FB can induce the cytokine response, we tested individual components of the FB: metacresol, mannitol, sodium acetate and glacial acetic acid. We found that almost all components of the FB can contribute to the induction of certain cytokines, such as PGE-2 and IL-8 (Figure 2). However, some components may have a greater contribution to the response. For example, it is interesting to note that for IL-8 (Figure 2b), a chemokine usually associated with inflammation, levels increased as much as four-fold due to the presence of mannitol.

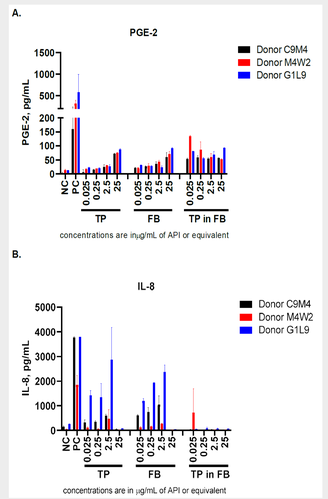

Figure 1. Formulation buffer is responsible for Prostaglandin-E2 and Interleukin-8 cytokine response in Teriparatide. PBMCs from three healthy donors were used to quantify induction of (A) PGE-2 and (B) IL-8 in response to TP diluted in PBS, FB diluted in PBS, and TP diluted in FB. Each bar shows a mean response and a standard deviation (N=3).

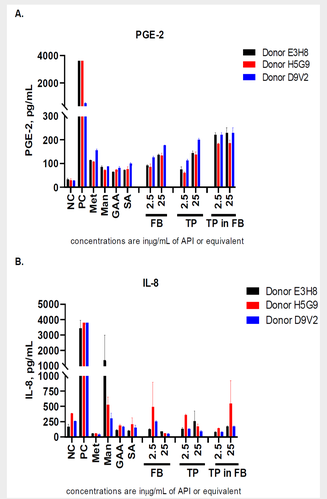

Figure 2: All components of formulation buffer of teriparatide induces A) prostaglandin-E2 (PGE-2) and B) interleukin 8 (IL-8) release. metacresol (Met), mannitol (Mann), sodium acetate (SA) and glacial acetic acid (GAA). Each bar shows a mean response and a standard deviation (N=3).

Handling of PBMC and Its Effects

During our investigation, we noticed that cytokine induction and assay sensitivity can vary greatly depending on PBMC storage and handling. To understand how the handling and processing of PBMC would affect the assay performance, we tested various storage and testing conditions, as shown in Table 1. We found that in general, the use of fresh PBMCs or PBMC that were within 4 hours of collection when they were cryopreserved resulted in the best cell recovery and viability and eventually resulted in the most sensitive platform to assess IIRMIs (Figure 2). Cultured and cryopreserved PBMCs, and fresh whole blood (prior to processing) may be used as some cell viability is retained. The use of refrigerated whole blood for 24 or 48 hours are not optimal because of the poor PBMC yield (with less than 10% cell viability remained) and possibly affected cellular responses.

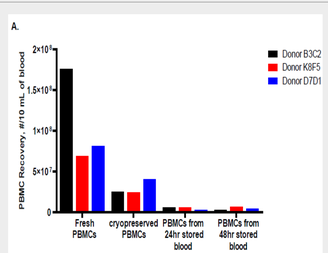

Figure 2: The effect of storage condition on PBMC cell recovery (A) and cell viability (B). PBMCs from three healthy donors were examined after fresh isolation, cryopreservation, and isolation from refrigerated blood (24 or 48 hours). Cell viability was then assessed using Acridine Orange/Propidium Iodide (AO/PI) staining. (A) Number of PBMCs recovered under the various storage/handling conditions. (B) Viability of stored PBMCs compared to their freshly isolated PBMC counterparts. Each bar shows the mean result and standard deviation of three independent samples (N=3)

CONCLUSIONS

We examined some of the best practices for conducting and optimizing the PBMC assay, and factors that could potentially affect cell viability and responses to support an IIRMI evaluation of generic peptide products. The differences in formulations and cell handling conditions can greatly affect cell survival and cytokine releases, which in turn affects the assay performance. Therefore, it is important for drug developers to investigate and understand how these factors would interfere with the cell-based assays during assay development and validation as this can also demonstrate whether the formulation interference is acceptable for the chosen cytokine signal.

From this study, we conclude that the use of fresh PBMC or PBMCs that were within 4 hours of collection before they were cryopreserved would yield the best results. The use of refrigerated whole blood (for up to 48 hours) is not advisable as it may significantly affect cellular response. In general, storage and handling conditions should be validated and justified.

Not all cytokine signals responds to IIRMIs equally. Therefore, it is important to assess which cytokines should be included in the panel readout in order to cover a wide range of IIRMIs and ensure optimal consistency between various donors is obtainable. For teriparatide, we found that a panel consist of minimally three cytokines would be able to provide at least one positive result for all ten IIRMIs (listed in Table 2) and these include: 1) IL-1 (or MIP-1), IP-10, and IL-8; or 2) IL-1 (or MIP-1), MCP-1 and IL-8 (or IL-6).

Finally, we would like to acknowledge that to adequately conduct and validate non-clinical immunogenicity assays can be challenging since there is a lack of standardized approaches and best practices for these non-clinical immunogenicity assays. We encourage the continued collaboration between regulatory, industry and academia in researching and developing standardized methods to these in vitro assays.

ACKNOWLEDGEMENTS

This research was supported by federal funds from the U.S. Food and Drug Administration, Center for Drug Evaluation and Research/Office of Generic Drugs, under an Inter-Agency Agreement with NCL/NCI/NIH (IAA #224-19-3008S). Dr. Liao was supported by an appointment to the Oak Ridge Institute for Science and Education (ORISE) Research Participation Program at the CDER administered by the ORISE through an agreement between the U. S. Department of Energy and CDER.

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1. FDA's guidance for industry, *ANDAs for Certain Highly Purified Synthetic Peptide Drug Products That Refer to Listed Drugs of rDNA Origin* (May 2021) https://www.fda.gov/media/107622/download
2. Holley, Claire K., et al. "An In Vitro Assessment of Immunostimulatory Responses to Ten Model Innate Immune Response Modulating Impurities \and Peptide Drug Product, Teriparatide." Molecules 26.24 (2021): 7461.

Opportunities with FDA's Office of Generic Drugs (OGD)

Looking for a post-doctoral opportunity?
https://www.zintellect.com/Opportunity/Details/FDA-CDER-2022-0791
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