Table 8: Structural MS – Best Practices for Predicting, Elucidating, and Monitoring Hotspots by MS

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Table Scope:

With its invaluable insights into the binding interfaces, conformational changes, and dynamic interactions that underpin biological processes, MS has become a crucial tool in understanding the fine aspects of biomolecular interactions. A deeper understanding of molecular complexities results from the skillful application of MS techniques, which enable researchers to predict and understand binding interfaces, conformational alterations, and dynamic interactions. This is of utmost importance in the field of Drug formulation because it makes it easier to identify and analyze key hotspots that control the efficacy, stability, and safety of pharmaceutical compounds. Mass Spectrometry provides significant insights into the dynamics of binding between Therapeutic compounds and their targets. MS-driven structural attribute elucidation reveals conformational variations that affect therapeutic functionality, resulting in robust drug design. The ability of MS to monitor in real-time also makes it possible to see dynamic interactions, which helps to improve drug development methods. This roundtable discussion will focus on the opportunities and best practices for predicting, elucidating, and monitoring hotspots for insights into biotherapeutic drug characterization and development.

Discussion Notes:

1. **Is the hotspots and post-translational modification (PTM) the same?**

   Hotspots and PTMs are related concepts but not the same. Hotspots refer to a specific region or sites on a protein that maybe particularly susceptible to chemical or environmental modification. In monoclonal antibody analysis, hotspots often refer to CDR regions modification. While, PTMs are biochemical process that happens in cells, altering their structure and function to regulate various cellular processes. Common PTMs include phosphorylation, glycosylation, acetylation, methylation, and so on. So for example, glycosylation is a not hotspot, but a PTM.

2. **How do researchers predict hotspots based on a molecule's information?**
   - Current hotspot prediction are largely for antibodies.
   - When there is no antibody structure available, researchers can use motif-based prediction replying on the primary structure of the antibody and prior knowledge of susceptible motifs.
   - If the structure is available, the secondary structure can be extracted and used to predict hotspots. Structure-based hotspot prediction involves using computational methods or observation from large structure database to identify regions likely to have modifications.

- Accurate prediction of hotspots is still quite challenging and requires more experimental MS data as well as crystal structures to build a comprehensive database.

3. **Elaborate on the scientific methods and experiments employed to characterize these hotspots in products**

   The traditional approach to characterize hotspots in products is LC-MS based peptide mapping method. The data generated during these experiments can be processed using both manual and software-based methods. However, it is important to note that artifacts may arise during LC-MS analysis, often related to sample preparation or data acquisition. Therefore, manual confirmation of the data is still necessary to ensure the accuracy of hotspot characterization.

4. **How to define a hotspot? What should we do after finding a hotspot?**

   Defining a hotspot involves considering several factors, including (i) type of protein (ii) type of hotspots, and (iii) impact on potency assays.

   (i) The characterization of a hotspot can vary depending on the type of protein and specific modification. For instance, hotspots on a vaccine may have less impact and be less critical compared to other antibody type proteins.
   (ii) Different modification may require different acceptance criteria. For example, for some modifications like % disulfide bond scramble, the acceptance criteria might be set at less than 1%, while for other modifications, it could be no greater than 5%.
   (iii) To confirm the impact of hotspots, in vivo bioassay studies may be necessary, especially when hotspot s are present at high levels.

   Overall, development group should thoroughly report their finding regarding hotspots. If the hotspots levels are high, even if they don't significantly affect bioactivity, design group should consider molecule redesign to maintain product consistency

5. **What are the key challenges faced when developing tools for real-time monitoring of hotspots, especially during the process development stage**

   MAM can be used as a real-time monitoring tool for hotspots analysis. While implementation of MAM can be complex and requires specialized expertise. The challenges of implementation of MAM include but not limited to data process complexity, instrumentation precision, and method

validation. In addition, some hotspots may not be detected through regular peptide mapping, posing a challenge. To address this, different fragmentation technique and multi-enzyme digestion can be employed. For major hotspots, a recommended approach for hotspots analysis involves a step-by-step process, starting with intact analysis, then subunit, and finally conducting peptide mapping.

6. What's the future of hotspot analysis?

The future of hotspots analysis is expected to involve the development of user-friendly mass spectrometers and software that require minimum training and human intervention during operation. This advancement aims to streamline the process of hotspot analysis, making it more accessible and efficient for research and professionals from various field or with limited experience.