Table 5: Structural MS – Best Practices for Predicting, Elucidating and Monitoring Hotspots by MS

Facilitator: Dennis Gessmann, *Pfizer Inc., Andover, MA, USA*
Scribe: Michelle English, *Protein Metrics Inc., Philadelphia, PA, USA*

Scope:
As early pipelines are filled with a greater diversity of therapeutic protein modalities aimed at ever more challenging targets an early understanding of potential liabilities is becoming increasingly important. Characterizing molecular suitability from many perspectives including manufacturing and storage for both drug discovery and development as well as providing product characterization in support of IND's, and BLAs is essential. A robust awareness of potential hotspots, such as amino acid modifications, truncations or aggregation serves as the basis for well-designed process and product quality attribute (PQA) monitoring of biotherapeutics. While often the goals it to remove hotspots from the molecule during engineering, some must remain to preserve activity and must be monitored throughout development, storage, and pharmacokinetic studies. This roundtable discussion will focus on the opportunities and best practices for predicting, elucidating, and monitoring hotspots for insights into structure-function-relationships and PQA assessments.

Questions for Discussion:
1. Prediction of Hotspots:
    a. How are physiochemical hotspots are predicted from primary structure.
    b. What scientific approaches and product characterization experiments are utilized?
2. Elucidation and Monitoring:
    a. How is elucidation vs monitoring being performed?
    b. How were orthogonal methods used for hotspot analysis?
    c. Discuss challenges in building monitoring tools during process development.
    d. How was hotspot monitoring used to support control strategies?
3. Physiochemical Hotspots of Interest:
    a. Discuss specific examples of notable modifications.
    b. Are there any modifications in certain regions linked to structure/function relationship or potency changes?

Discussion Notes:
Prediction of Hotspots:

One example used a tiered ranking system of potential sequence liabilities
Tier 1 – Serious, would not let molecule go forward
Tier 2 – Concern but program can progress – anything in CDR
Tier 3 – Flag and monitor
With quarterly reviews of hotspots being monitored. New hotspots may come from literature or evaluations of solvent accessibility

A question was asked about identifying hotspots to be evaluated; Is there an online tool that you might use to identify potential hotspots from sequence. None that the team knew of though some disulfide tools are available.

Most organizations develop their own internal prediction tools. Some even have multiple tools available as the result of mergers and they all seem to work well internally. Most internal tools are highly focused on CDRs. Recent publications highlight how in house data might be mined to create repositories of sequence liability and alignment information which might be used for prediction (eg Jacobitz et al. AAPS Open (2022) 8:10 https://doi.org/10.1186/s41120-022-00057-2).

As solvent accessibility can affect the propensity for a potential liability to become an actual liability, a question was asked if the sequence location of a methionine might affect its solvent accessibility and therefore ability to oxidize. One study shows that solvent accessibility really does have a big contribution (Journal of Pharmaceutical Sciences 107 (2018) 1282-1289).

Overall, it sounds like there is no central location for sequence liability information, it is more a collection of 'tribal knowledge'.

Given the assertion that MetOx is heavily affected by solvent accessibility, a question was asked if anyone had ever used FPOP to evaluate methionine hotspots? The answer was essentially no though it is an interesting approach however since it is a different mechanism of oxidation, it may not be directly applicable.

Elucidation and Monitoring:

The primary approach is forced degradation with peptide mapping. For example: High pH, Low pH, chemical oxidation and light exposure.

This method is used by many organizations and can serve several purposes

- Developing methods
- Feedback to research
- Feedback to release assays to make sure they can be identified in the selected assays

Is middle down used in every organization? Generally, yes though in some, only if the peptide mapping shows something requiring further investigation.

Is monitoring different when the target is not a mAb? For example, in the context of an fc fusion protein, is everything 'CDR'. What is different if the product is harvested rather

than manufactured. The guidance is much more based on potency rather than structure elucidation for these types of products.

Working with ADCs introduces many more considerations as the construction and stability of the drug molecule can be more important that sequence liabilities. Drug preservation becomes the most important issue. One example highlighted was that while pH5 may be fine for the molecule, it may harm the drug linkage and have an outsized affect.

For receptor proteins, it is important to find binding sites and assure there are no issues there. However, this needs to be tested as literature about binding sites is not always an accurate predictor of the behavior of a sequence liability in these areas. An example was given regarding significant forced oxidation at a putative binding site that had no effect on potency.

Regarding assays used throughout the process, often elucidation and monitoring are one in the same, not actually two different assays. The information gathered in one phase can then be directly utilized in later phases. However, this must be done with an awareness of downstream conditions as they might differ.

Depending on project phase different activities might be required. Often later stages apply MAM type assays. And while the forced deg study informs the attributes being monitored, the presence of MS1 provides the data for deeper investigation when needed.

Manufacturability assessment may also differ regarding what is needed. So in early process stages intact mass may play a more important role. In this context Intact and reduced may provide orthogonal information.

While most organizations use SPR later in development processes, several organizations use it early to determine if a hotspot really matters and should eliminate candidates. However, others wondered whether even though the hotspot may not matter, does it still possibly indicate a process that is not fully in control and would you want to deal with that in a late stage or commercial environment?

Challenges to developing assays include equipment standardization. For example, what if a downstream lab does not have the instruments capable of seeing an isomerization that was identified in an upstream lab? Additionally, failure to align methods can cause similar disjunctions between internal organizations. One commenter suggests reading the book "How NASA Builds Teams" to see ways to deal with these types of organizational issues.

One attendee noted that this type of method difference does not only happen across organizations, but often can be seen when working on both late and early-stage projects at

the same time. This happens as platform methods simply do not work for every molecule under development at a given point in time.

Like SPR, in vivo stability studies do not happen consistently across companies. Some do in vivo studies very early, while others wait until later stages of development. Though many more do 'ex vivo' or closed plasma stability studies as early as research. While this is important it was still noted that this does not always accurately reflect what will happen in later in vivo studies.

Returning to an earlier topic, it was again noted that alignment of research and development is often a major pain point in many organizations. A specific example was related to tox studies where early exploratory tox studies show no issues but later GLP tox studies failed. The organization now needs to figure out why it failed. Some organizations try to put in semi-CMC style controls in there early-stage organizations as well to limit these types of deviations.

Physiochemical Hotspots of Interest:

A question was asked about troubling or unusual modifications.

While most are anticipating hydroxyproline, there are also hydroxylysine modifications that affect the product. Another had seen hydroxylysine on a linker that was then the target for glucosyl-galactosyl modification.

Another lab has seen hydroxyproline as a pervasive low-level modification in starvation situations. The interpretation was complicated by the presence of isomeric species though ETHCD can differentiate.

Another odd example was the addition of an ADC drug to a histidine residue in the fc.

Finally, there was an example of a non-tyrosine sulfation on an in licensed product.