NICEATM — *National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods*

ICCVAM — *Interagency Coordinating Committee on the Validation of Alternative Methods*

Session 3A Panel Discussion Summary: Replacement Alternatives for Endopeptidase Assays

Dr. Susan Maslanka & Dr. Shashi Sharma, Moderators

ICCVAM/NICEATM/ECVAM Scientific Workshop on Alternative Methods to Refine, Reduce, and Replace the Mouse LD_{50} Assay For Botulinum Toxin Testing

November 13 -14, 2006

Crowne Plaza Hotel, Silver Spring, MD

National Toxicology Program | NIEHS | National Institutes of Health | U.S. Department of Health and Human Services

Panel Discussion Question # 1

- **Recognizing that it will be necessary to establish that alternative methods are appropriate for each particular pharmaceutical product, can any of the current endopeptidase methods be used now to *replace* animals for potency testing of botulinum toxin? If no, what limiting factors prevent these methods from being used as a replacement for the mouse LD_{50} assay?**
  - The consensus is that the assay cannot currently be used to replace animal testing
  - Given that the endopeptidase assays are not a complete measure of the biological activities of the botulinum neurotoxins, it is debatable whether these assay could completely replace the mouse LD50 test unless performed in conjunction with another *in vitro* assay (e.g., receptor binding)
  - These assays are more likely to overestimate potency, compared to the LD50 assay
  - Limitations include:
    - Very dependent on assay conditions (e.g., pH, sample matrix)
    - Only measures light chain activity
    - May only be used for specific conditions
    - May not be useful for product stability studies

Panel Discussion Question # 2

- **Based on the needs for detecting botulinum toxin in environmental or biological samples (e.g., speed, portability, throughput) as discussed in Session 1, could the endopeptidase assays discussed be used to *replace* animals for these kinds of samples? If no, what limiting factors prevent these methods from being used as a replacement for the mouse LD_{50} assay?**
  - The consensus is that these assay cannot currently be used as a replacement
  - Limitations include:
    - Sample preparation in complex matrices and potential assay interference
    - Clean-up step is needed to remove such interference
    - Botulinum toxin endopeptidases require a controlled buffer environment for optimal expression of their enzymatic activities

Panel Discussion Question # 3

- **Can any of the current endopeptidase methods be used now to *reduce* the number of animals used for potency testing of botulinum toxin? If no, what limiting factors prevent these methods from being used to reduce the number of animals used in the mouse LD_{50} assay?**
  - In principle, it should be possible to use an endopeptidase assay to estimate the concentration of botulinum neurotoxin in a preparation intended for pharmaceutical use.
    - This would reduce the number of animals needed in the LD50 test by identifying a relatively narrow dose range, based on the toxin concentration indicated by the endopeptidase assay
    - Must be appropriately validated for this purpose and for each product

Panel Discussion Question # 4

- **Based on the needs for detecting botulinum toxin in environmental or biological samples (e.g., speed, portability, throughput) as discussed in Session 1, could the endopeptidase assays discussed be used to *reduce* the number of animals used for these kinds of samples? If no, what limiting factors prevent these methods from being used to reduce the number of animals used in the mouse LD_{50} assay?**
  - The endopeptidase assays could be used for screening of large numbers of a food matrix previously known and/or validated to identify a specific botulinum toxin type or subtype.
    - LD50 assays could then be used to test only endopeptidase positive samples
  - An endopeptidase assay, in parallel with an LD50 assay, may eliminate the need for neutralization studies in preidentified toxin-contaminated matrices, which would result in an immediate reduction in the number of animals.
  - There is a need for clean-up - antibody capture step. So, this method is limited to the detection of known toxin types and subtypes in preidentified sample matrices.

Panel Discussion Question # 5

- **Should endopeptidase methods other than those discussed so far during this workshop be considered for development and validation for potency testing or detection of botulinum toxin?**
  - None were identified

Panel Discussion Question # 6 (1)

- **What are the pros and cons of the different endopeptidase methods reviewed?**
  - Cons include:
    - Sensitivity, specificity, robustness and transferability will vary with assay formats
    - May be susceptible to endogenous proteases in complex matrices
    - Cost and availability of reagents are critical factors and assay formats relying on antibodies (monoclonal, or site directed) will encounter problems if long-term supply of high quality reagents cannot be guaranteed.
    - Sample preparation
    - Lacks two aspects of toxin's functional activity, (e.g., binding and translocation)
    - M.S. platform is expensive
    - Need for antibody to remove toxin from matrix limits detection to known toxin types and possibly subtypes

Panel Discussion Question # 6 (2)

- **What are the pros and cons of the different endopeptidase methods reviewed?**
  - Pros include:
    - Use of fluorescent substrates is likely to be more attractive to manufacturers, because these methods provide potentially more robust and high precision assays
    - fluorescence-based assays are one-step assays; more steps increase variability
    - M.S. platform has high throughput capacity
    - M.S. platform has enhanced specificity based on mass of cleavage products of substrate

Panel Discussion Question # 7

- **What current knowledge gaps with regard to the reviewed endopeptidase methods must be addressed to further their use in potency testing or detection (as discussed in Session 1) of botulinum toxin? What additional studies are needed?**
  - Unknown subtypes of serotypes
  - Substrate production methods should be optimized
  - It is critical to know what each assay format can or cannot do and how it can be adapted for a particular application before initiating validation studies
    - For each and every intended purpose, validation is required specifically designed to “fit for purpose”

Panel Discussion Question # 8

- **Of the endopeptidase methods discussed, which should have the highest priority for further development and validation studies?**
  - No consensus
  - This is largely dependent on intended purpose of the assay, which will have to be carefully defined prior to design of the validation process
  - For each method, validation criteria should be established within the laboratory

Panel Discussion Question # 9

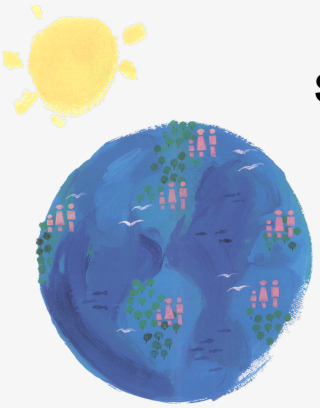
- **What are the essential characteristics of an endopeptidase method sufficient to replace or reduce the number of animals used for potency testing or detection (as discussed in Session 1) of botulinum toxin?**
  - For an assay to replace the mouse bioassay, several criteria would have to be met:
    - The assay would have to be sensitive (i.e., close to, or better than the mouse test)
    - Assays should detect all toxin sub-types (e.g., for BoNT/A, the assay should detect all four subtypes A1 – A4 with the desired sensitivity)
    - The sample media should not affect assay sensitivity - matrix effects
    - The assay should take less than 5 hr to perform
    - The cost of performing the assay should not be prohibitive
    - The results should be reproducible

Panel Discussion Question # 10

- **What is the best way to assess the validation status of these endopeptidase methods?**
  - This is largely dependent on the intended purpose of the assay, which will have to be carefully defined prior to design of validation process
  - Multilaboratory (i.e., “round robin”) testing is needed to determine robustness
  - Protocol for validation must be developed by the method developer

NICEATM — *National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods*

ICCVAM — *Interagency Coordinating Committee on the Validation of Alternative Methods*



Session 3B Panel Discussion Summary: Replacement Alternatives for Cell-based Assays

Dr. Susan Maslanka & Dr. Shashi Sharma, Moderators

ICCVAM/NICEATM/ECVAM Scientific Workshop on Alternative Methods to Refine, Reduce, and Replace the Mouse LD_{50} Assay For Botulinum Toxin Testing

November 13 -14, 2006

Crowne Plaza Hotel, Silver Spring, MD

National Toxicology Program | NIEHS | National Institutes of Health | U.S. Department of Health and Human Services

Panel Discussion Question # 1

- **Recognizing that it will be necessary to establish that alternative methods are appropriate for each particular pharmaceutical product, can any of the current cell-based methods be used now to *replace* or *reduce* animals for potency testing of botulinum toxin? If no, what limiting factors prevent these methods from being used as a replacement for the mouse LD_{50} assay?**
  - While there is potential, no methods could currently replace or reduce animal use for potency testing
  - Ease of use, sensitivity, robustness, transferability as well as precision and reproducibility may be difficult with cell-based assay at this stage
  - More data is needed
  - Cell line variability among different assays
  - Shelf life of cell line
    - However, lack proper synapses and glia, so not a functional assay which could be misleading

Panel Discussion Question # 2

- **Based on the needs for detecting botulinum toxin in environmental or biological samples (e.g., speed, portability, throughput) as discussed in Session 1, could the cell-based assays discussed be used to replace animals for these kinds of samples? If no, what limiting factors prevent these methods from being used as a replacement for the mouse LD_{50} assay?**
  - Same comments as EP assays (i.e., matrix affects)
  - Sensitivity relative to LD50 is an issue
    - Cell-based assays are an order of magnitude less sensitive than the LD50 test
  - May be useful in another application - identification of small molecule inhibitors or antibodies, where sensitivity isn't as large a concern

Panel Discussion Question # 5

- **Should cell-based methods other than those discussed so far during this workshop be considered for development and validation for potency testing or detection of botulinum toxin?**
  - "Neuronal Network Biosensors," because they provide cellular responses using the sensitive electrophysiological mechanisms that are directly affected by BoNT action

Panel Discussion Question # 6

- **What are the pros and cons of the different cell-based methods reviewed?**
  - Cons:
    - Very unpredictable
    - Sensitivity a problem - most work only conducted using purified toxin
    - Variability relative to LD50 test not known
    - Unknown which cell types and/or cell lines are the best
    - Multiple cell lines which may more closely mimic the mouse may be too complex for uniform adoption in many laboratories
  - Pros:
    - Cell based methods may be best option in terms of capturing all mechanisms of intoxication - binding, translocation, enzymatic activity

Panel Discussion Question # 7

- **What current knowledge gaps with regard to the reviewed cell-based methods must be addressed to further their use in potency testing or detection (as discussed in Session 1) of botulinum toxin? What additional studies are needed?**
  - Co-culture methods should be explored
  - There needs to be a better understanding of motor neuron differentiation
  - pH effects on cell lines should be explored
  - Not sure how close to physiological situation is needed?
  - More characterization of binding effects, how receptors are expressed, how sensitive to environmental effects, etc. is needed
  - Although all synapses are eventually attacked by BoNT-A, there appears to be a quantitative difference between different types of synapses
    - May depend primarily on the size of the presynaptic membrane

Panel Discussion Question # 8

- **Of the cell-based methods discussed, which should have the highest priority for further development and validation studies?**
  - Single cell line may initially be the “easiest” approach to develop, standardize, and validate
  - Multiculture approach should also be pursued in order to strive toward total mouse replacement

Panel Discussion Question # 9

- **What are the essential characteristics of an cell-based method sufficient to replace or reduce the number of animals used for potency testing or detection (as discussed in Session 1) of botulinum toxin?**
  - Need to measure both inhibition of release and cleavage of the substrate
  - Need to be standardized, easy to maintain, easily transferable
  - Need to be rapid, especially in the context of bioterrorism or naturally occurring food borne outbreaks
  - Need to be as sensitive as the mouse bioassay and show a reproducible correlation between activity and mouse LD50 units
  - Must not exhibit matrix effects
  - The results must be reproducible when done by different operators of varying skill levels in different laboratories
  - The ideal cell-based assay would use an immortalized cell line instead of primary cultures

Panel Discussion Question # 10

- **What is the best way to assess the validation status of these cell-based methods?**
  - Need reference materials
    - May need different reference materials for different licensed products
  - Which toxins are going to be used? Which subtypes?
  - Need to validate for specific purpose (i.e., detection, potency, antitoxin)