

## Organophosphate mechanism of action and potential biomarker studies

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## Topics

1. A new motif of organophosphorus agent (OP) binding to tyrosine in proteins that have no active site serine
2. OP binding to Tubulin in brain as a possible explanation for OP neurotoxicity
3. Antibodies for diagnosis of OP exposure and for isolation of OP-labeled proteins

## Background

- Inhibition of acetylcholinesterase explains the acute toxicity of organophosphorus agents (OP) but does not explain low dose toxicity.
- Epidemiologists have linked low dose exposure to Parkinson's disease, neurologic disorders, and depression.

## Mechanism of low dose toxicity is unknown

- These illnesses result from OP doses that do not inhibit acetylcholinesterase activity.
- A mechanism to explain low dose toxicity is needed.

## Hypothesis

- Our hypothesis is that unknown proteins are modified by organophosphorus poisons and that the modification disrupts the function of the nervous system.

## Mass spectrometry to test our hypothesis

- We are using mass spectrometry to identify proteins covalently modified by organophosphorus agents and to identify the modified amino acid.

## Testing our hypothesis

- Live mice treated with OP
- Mouse tissues treated with OP
- Purified proteins treated with OP
- Plasma from humans hospitalized for pesticide poisoning

## Result: OP-labeled proteins

- Serine hydrolases.  
Acetylcholinesterase, Butyrylcholinesterase, Carboxylesterase, Acyl peptide hydrolase, Prolylendopeptidase, Lysophospholipase
- No active site serine.  
Albumin, Tubulin, Kinesin, Alpha-2-macroglobulin, Complement component 3, Transferrin, Alpha-1-antitrypsin, Apolipoprotein, Adenine nucleotide translocase-1, ATP synthase beta subunit

## Covalent binding to Tyrosine is a new OP binding motif

- The OP attachment site in albumin, tubulin, and in other proteins is tyrosine.
- **We conclude that OP binding to tyrosine is a new motif for OP modification of proteins that have no active site serine.**

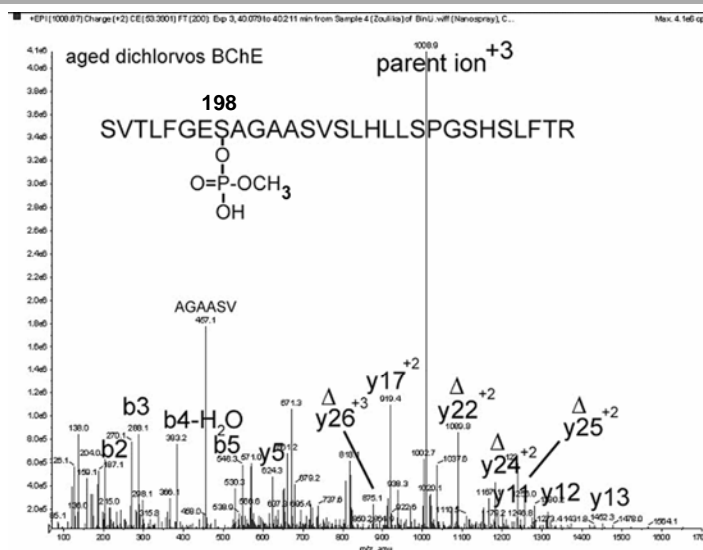
## Testing plasma from people exposed to OP

- Plasma was obtained from the Poison Center in Omaha, Nebraska and from the Paris police.
- Methods were developed to identify OP adducts on butyrylcholinesterase and on albumin in human plasma.

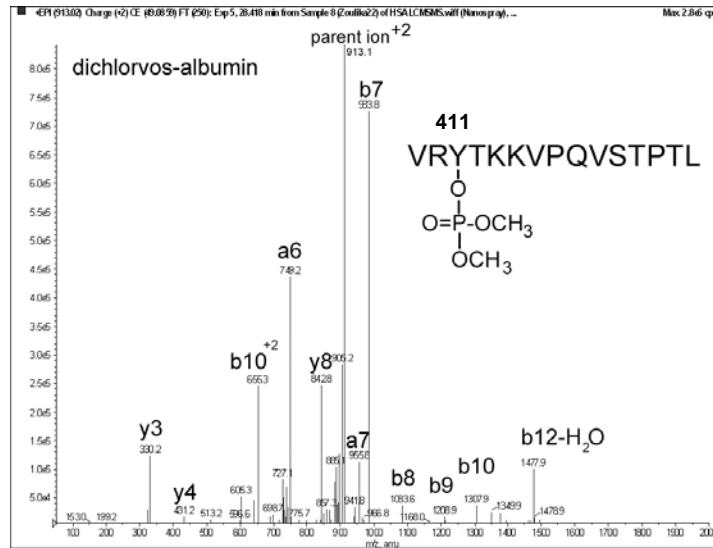
## Mass Spectrometry Result

- Plasma from dichlorvos poisoned patients contains dichlorvos-labeled butyrylcholinesterase (on Ser 198) and dichlorvos-labeled albumin (on Tyr 411).
- OP-labeled butyrylcholinesterase was expected.
- The finding of OP-labeled albumin in patient plasma is a novel result.

## MSMS spectrum for dichlorvos labeled butyrylcholinesterase in patient plasma



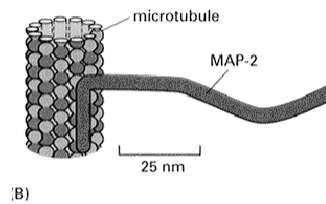
## MSMS spectrum for dichlorvos labeled albumin from patient



## Significance (Take home point)

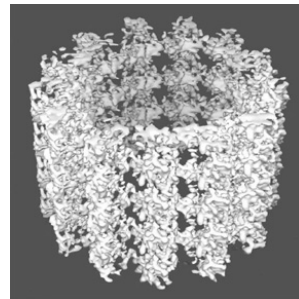
- The presence of OP-labeled albumin in humans suggests that other proteins can also become labeled on tyrosine by OP.
- OP-labeled albumin does not explain neurotoxicity.
- **The search for proteins involved in OP toxicity is not limited to proteins in the serine hydrolase family.**

## Topic 2. Tubulin in brain could be involved in OP neurotoxicity



### Focus on tubulin

- Tubulin was selected for detailed study because tubulin is modified by OP on tyrosine when mouse brain extracts are treated with OP.

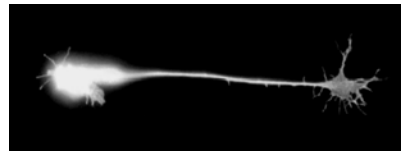


Tubulin polymer makes hollow microtubules

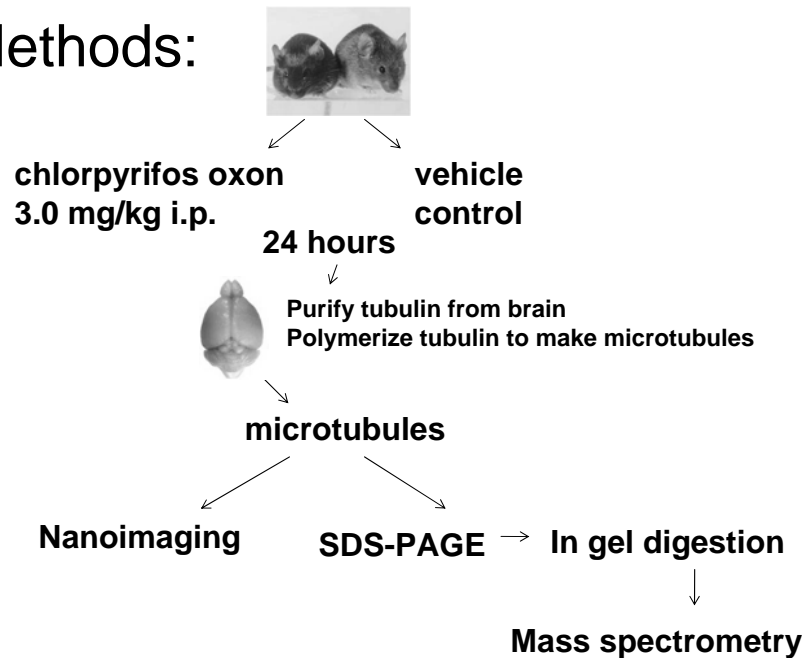


## Focus on tubulin

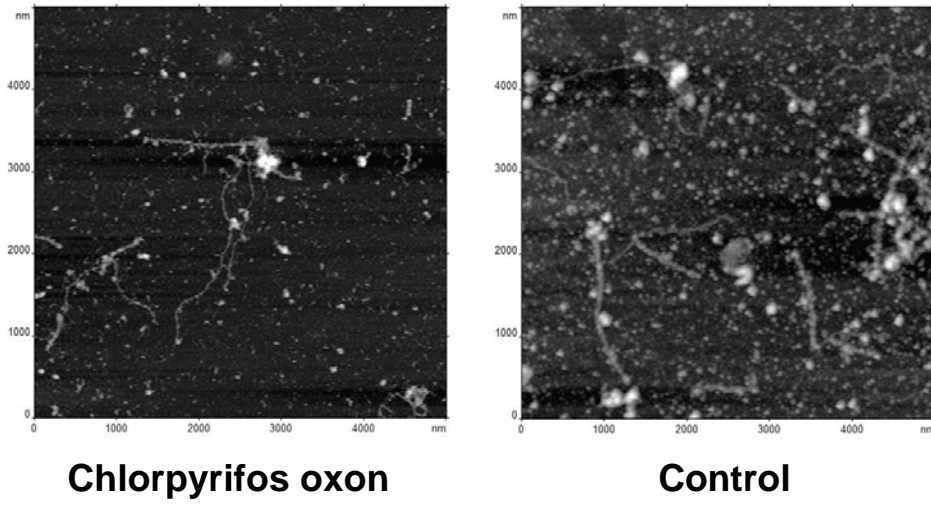
- Tubulin has important functions in neurons.
  - Tubulin polymerizes to form microtubules. Microtubules are the highway for transport of cell components from the cell body to the nerve axons.
  - Disruption of microtubule function results in cell death.



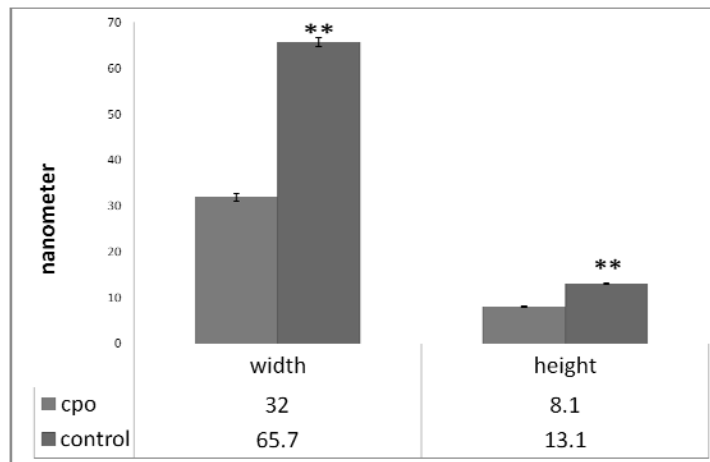
## Methods:



## Result: Nanoimages of microtubules



### Microtubule width and height comparison



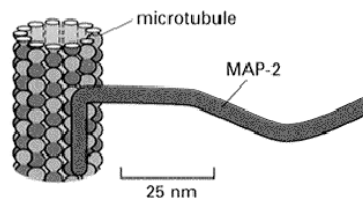
Microtubule height and width were measured for 150 fibers at 4 to 5 positions along the fiber (a total of 700 measurements)

## Nanoimaging results

- Tubulin from OP treated mice assembles into abnormally thin microtubules.
- The microtubules from OP treated mice have fewer microtubule-associated proteins.

## Microtubule-associated proteins

- Microtubule-associated proteins are essential for microtubule function
  - Facilitate polymerization and stabilize microtubule structure
  - Transport cargo to the nerve endings by traveling along the microtubules



Proteins are selectively depleted from  
microtubules of chlorpyrifos oxon treated  
mice

- Microtubule-associated protein 2 isoform 1
- Tau
- Myosin Va
- Cytoskeleton associated protein 5
- Heat shock protein 84 kDa
- Dynein
- Alpha-internexin
- Plectin 1 isoform 4

## Conclusion

**Exposure to chlorpyrifos oxon in vivo:**

- Disrupts the structure of microtubules.
- Depletes microtubules of the normal complement of microtubule-associated proteins.

## Impact (Take home point)

These results suggest microtubule function is impaired by OP exposure.

Dysfunctional microtubules could be part of the explanation for the neurotoxicity associated with exposure to OP.

## Impact (Take home point)

Proteins not yet identified could also be involved in OP neurotoxicity.

If these unidentified proteins are in low abundance, the OP-labeled versions will be difficult to detect by mass spectrometry.

The task of finding them will be easier if they can be immunoprecipitated with an antibody specific for OP-tyrosine.

## Topic 3

Antibodies for diagnosis of OP  
exposure  
and  
for isolation of OP-labeled proteins

### Background

- To date no one has made an antibody that detects nerve agent exposure.
- Attempts by others have focused on an antibody to nerve agent-modified cholinesterase. This strategy has failed because adducts on the active site serine of cholinesterase are unstable.
- We have a new strategy and we have preliminary results to suggest our new strategy works.

## New strategy

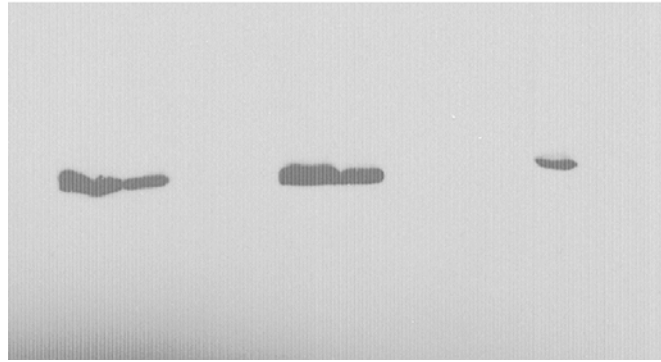
- Our new strategy is based on our mass spectrometry results. We have found that OP make a bond not only with the active site serine of cholinesterase, but also with tyrosine in albumin and tyrosine in other proteins.
- OP modified tyrosine is very stable.
- Animal studies have shown that nerve agent-labeled albumin can be detected as late as 24 days after exposure, at a time when no nerve agent modified cholinesterase is detectable.

## Polyclonal antibody results

- Polyclonal antibodies were made in rabbit to a small peptide labeled on tyrosine with soman.
- The antiserum was tested for binding specificity on Western blots.
- Binding affinity was tested in a competition ELISA assay.

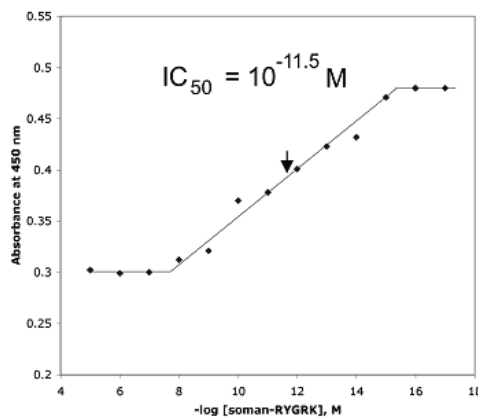
## Western blot result

Hu albumin      mouse albumin      human transferrin  
control 10 soman 5      control 10 soman 5      control 7 soman 7  $\mu$ g



The polyclonal antibody recognizes soman-labeled albumin and soman-labeled transferrin. It does not recognize unlabeled proteins. This result shows high binding specificity for soman-tyrosine adducts.

## Binding affinity result



Binding affinity was tested in a competition assay between soman-albumin and soman-peptide RYGRK for binding to the polyclonal antibody.

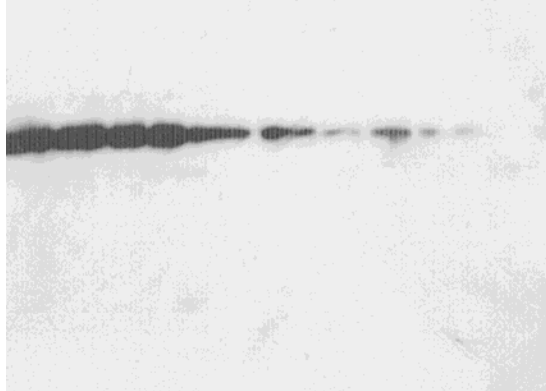
The high binding affinity is a very promising result.

The binding affinity of  $10^{-11.5} \text{ M}$  is a very promising



## Limit of detection

0.5 0.5 0.1 0.1 0.025 0.025 0.005 0.005  
0.25 0.25 0.050 0.050 0.01 0.01 ug soman-albumin



**Western blot**  
Lanes were loaded in duplicate with 0.5 to 0.005  $\mu\text{g}$  of soman-labeled albumin. The blot was hybridized with polyclonal antibody diluted 1:2000.

**The limit of detection was 0.01 micrograms of soman-labeled human albumin.**

Human plasma has 40,000 micrograms of albumin per milliliter.

Human saliva has 35 micrograms albumin per milliliter.

A limit of detection of 0.01 micrograms means that low exposure levels will be detectable.

## Plan

- The plan is to make monoclonal antibodies to a variety of OP-tyrosine.
- Each OP will have a specific antibody for that OP.
- Reasons for making monoclonals rather than polyclonals
  - monoclonals can be made in unlimited quantities
  - monoclonals have higher specificity
  - monoclonals can be labeled with a fluorescent tag to increase detection sensitivity

## Potential uses of the monoclonals

- Monoclonal antibodies will detect low dose exposure to OP. The antibodies can be used in hand-held devices.
- Pesticide workers, war-fighters, and the worried-well can benefit.
- Monoclonal antibodies can be used to isolate OP-labeled proteins and thus lead to an understanding of the mechanism of low dose neurotoxicity.

## Advantages of an antibody test for exposure

- Simple
- Rapid
- Inexpensive
- Portable

## Conclusion

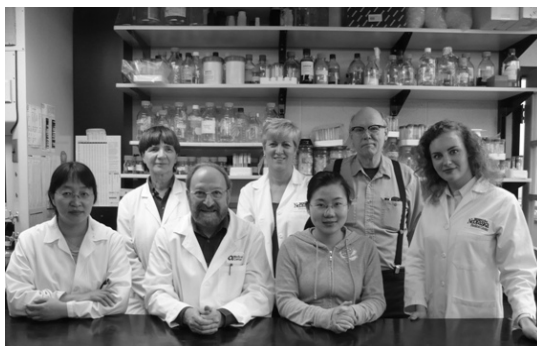
- Monoclonal antibodies for detection of OP exposure are expected to be specific and highly sensitive.
- Exposure levels too low to cause toxic symptoms should be detectable with the monoclonal antibodies.

## Conclusion (Take home point)

The novelty of our approach is that antibodies will be made against OP adducts on tyrosine.

The strengths are the solid evidence from mass spectrometry that OP make a covalent bond with tyrosine in proteins, and our demonstration that antibodies against soman-labeled tyrosine show high affinity and specificity.

## Acknowledgements



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