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# Understanding the actions of a dietary anti-oxidant at the protein and small molecule level using top-down proteomics, enzyme assays and mass spectrometry

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## OUTLINE

- I. Top-down proteomic analysis of actions of a dietary supplement, grape seed extract (GSE), in mammalian brain;
- II. FT-ICR MS mapping of reactive aldehyde 4HNE adduct sites on recombinant creatine kinase (CK-BB);
- III. Enzyme assays: HNE stoichiometrically poisons CK-BB;

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**Our principal goal: to understand the molecular basis of human chronic conditions/diseases, to develop prevention or therapies.**

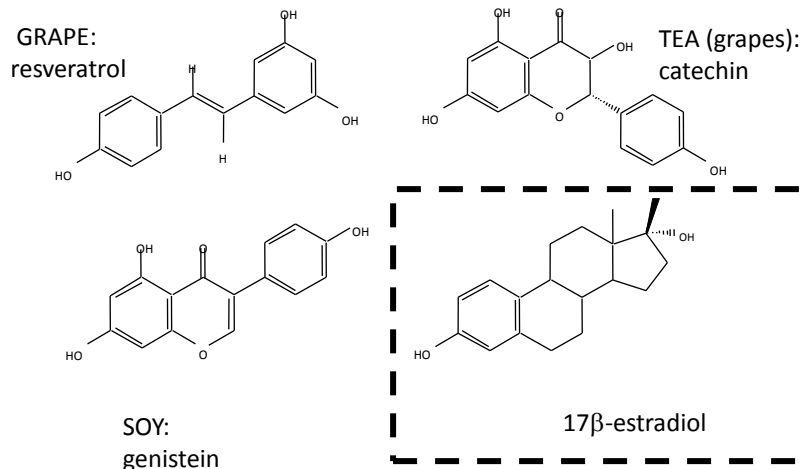
**Strategy: a proteomics approach**

**Hypothesis: Actions of “beneficial” agents such as dietary anti-oxidants in normal and disease tissue will reveal subproteomes of proteins “at risk” for disease-relevant changes.**

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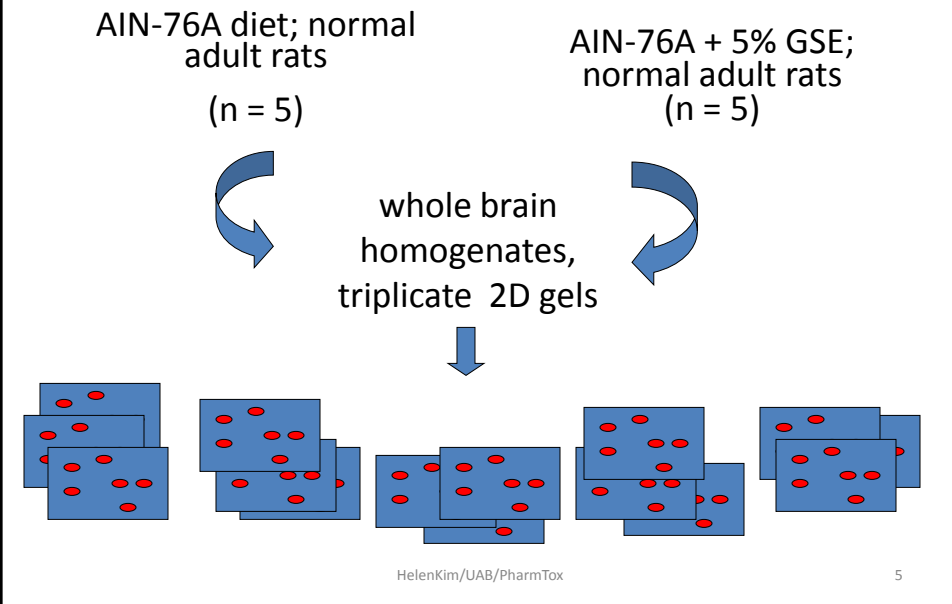
**POLYPHENOLS: similar structures among themselves, and with  $17\beta$ -estradiol**



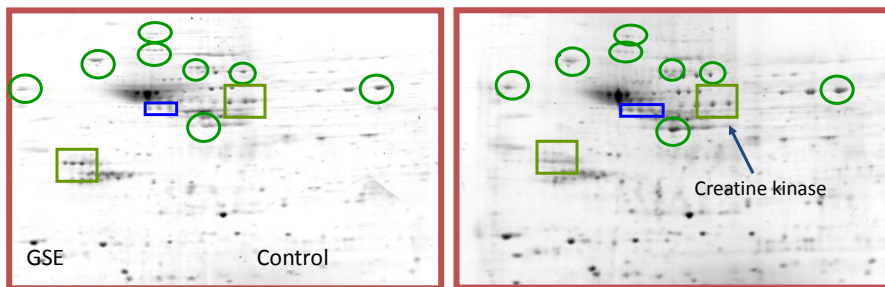
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## The experiment: give grapeseed powder in rat diet; examine brain proteomes



## Image analysis indicated rat brain proteins were affected by GSE.



- Different in intensity
- Different in horizontal position

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## Database of protein differences in GSE vs CT brains

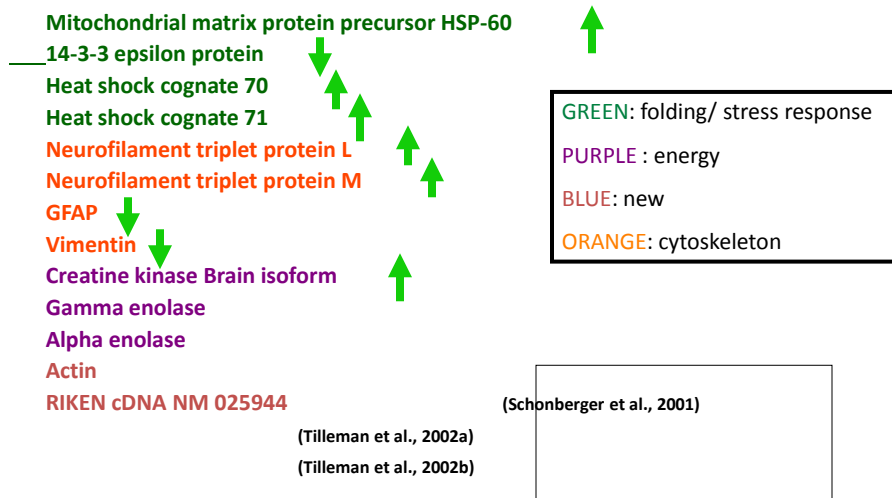
Protein Name	#matched pep	Accession #	MOWSE	Obs m.w.	Pred m.w.	Obs pl	Pred pl	Nature of change in GSE brains
Mitochondrial matrix protein precursor P60	10	P19227	*1.26E+04	64900	60956	5.6	5.9	+1.5
Creatine Kinase BB chain	12	P07335	*1.66E+05	45600	42712	5.45	5.3	+1.52 Translocation to Acidic pH
Actin	8	P10365	*2.18E+05	42000	41636	5.3	5.4	Less complex
GFAP	20	P47819	*9.67E+09	49000	49943	5.4	5.3	- 1.6
14-3-3 epsilon	10	P42655	*1.41E+09	31900	29174	4.49	4.6	- 2.1
Alpha Enolase	9	P04764	*6.64E+05	46000	46985	6.0	6.2	Less complex
Gamma Enolase	10	P07323	95	47000	47111	5.12	5.03	Less complex
RIKEN cDNA (NM 025994)	9	NP080270	169 95	26000 26000	25084 25084	5.0 5.1	5.0	-1.56
HSC-70	12	gi4103877	110	70321	42455	5.9	6.64	+1.63
HSC-71	16	gi123644	105	70386	71195	5.43	5.49	+1.91
Neurofilament L Triplet protein	14	gi13929098	120	61025	61298	4.61	4.63	+1.63
Neurofilament M triplet protein	19	gi8393823	153	95086	95591	4.75	4.76	+1.73
Vimentin	10	gi202368	93	53600	53641	5.09	5.06	-1.52

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(Deshane et al., 2004. J. Agric. Food Chem.)

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**Initial conclusion: GSE is neuroprotective, since its effects on proteins are counter to the directions of change for the same proteins in disease.**



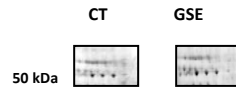
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(Deshane et al., 2004. J. Agric. Food Chem.)

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## Western blot analysis corroborated 2D gel image analysis quantitation

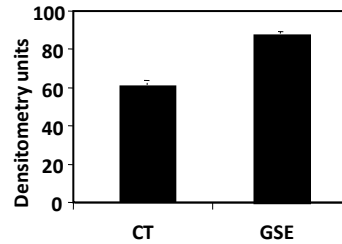
### A. Stained gel for HSP-60



### B. Western Blots



### C. Quantitative Densitometry

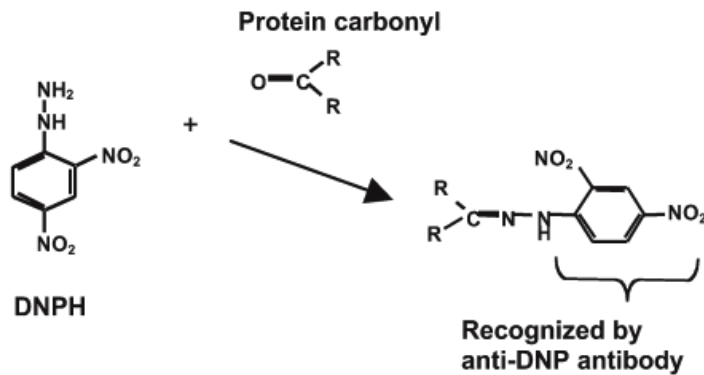


(Deshane et al., 2004. J. Agric. Food Chem.)

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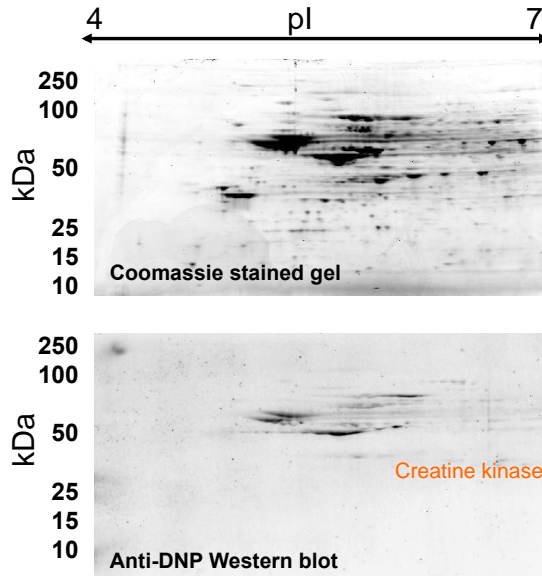
## Use chemistry to determine whether GSE affects oxidations



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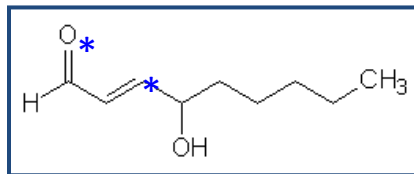
## Preliminary experiments indicated GSE affected oxidations of abundant proteins



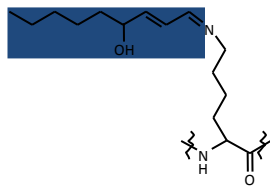
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## 4-hydroxy-2-nonenal (4HNE)

A reactive aldehyde resulting from arachidonic acid

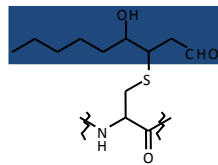


Reacts with K, C, and H



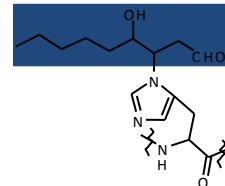
4HNE-Modified Lysine

Schiff Base Adduct



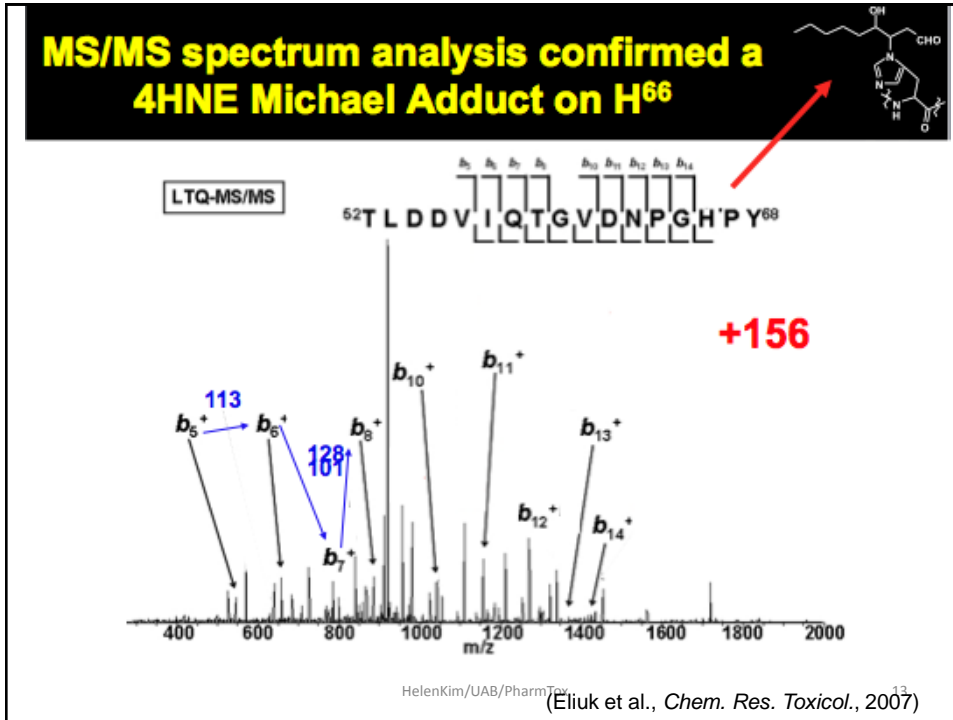
4HNE-Modified Cysteine

Michael Adduct



4HNE-Modified Histidine

Michael Adduct

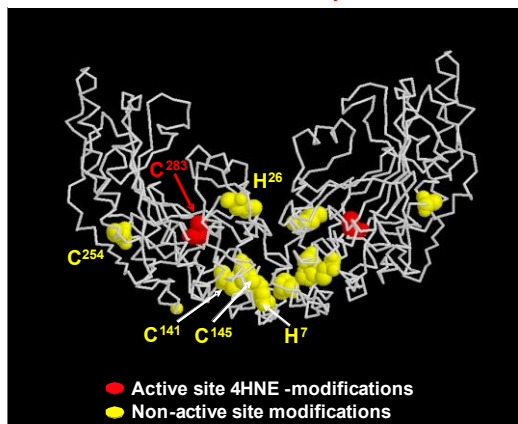


Modified Amino Acid	Concentration of 4HNE (μM)					
	5000	300	100	30	10	5
H <sup>7</sup>	M,S	M,S	M,S	S		
H <sup>26</sup>	M	M	M	M		
H <sup>29</sup>	M	M				
K <sup>45</sup>	M					
<b>H<sup>66</sup></b>	<b>M</b>	<b>M</b>				
K <sup>86</sup>	M	M				
H <sup>97</sup>	M	M	M			
K <sup>101</sup>	M	M				
C <sup>141</sup>	M	M	M	M		
C <sup>145</sup>	M	M	M	M		
K <sup>177</sup>	M					
<b>H<sup>191</sup></b>	<b>M</b>	<b>M</b>	<b>M</b>			
H <sup>219</sup>	M,S	M,S	M			
H <sup>234</sup>	M,S	M,S	S			
K <sup>247</sup>	M,S					
C <sup>254</sup>	M,S	M,S	M,S	M,S	M	M
H <sup>276</sup>	M	M				
<b>C<sup>283</sup></b>	<b>M</b>	<b>M</b>	<b>M</b>	<b>M</b>	<b>M</b>	
<b>H<sup>296</sup></b>	<b>M,S</b>	<b>M,S</b>	<b>S</b>			
H <sup>305</sup>	M	M				
K <sup>313</sup>	M					
K <sup>358</sup>	M					
K <sup>381</sup>	M					

**Sites of HNE-adducts on CKBB over a range of concentrations; RED indicates active-site residues**

(Eliuk et al., *Chem. Res. Toxicol.*, 2007)

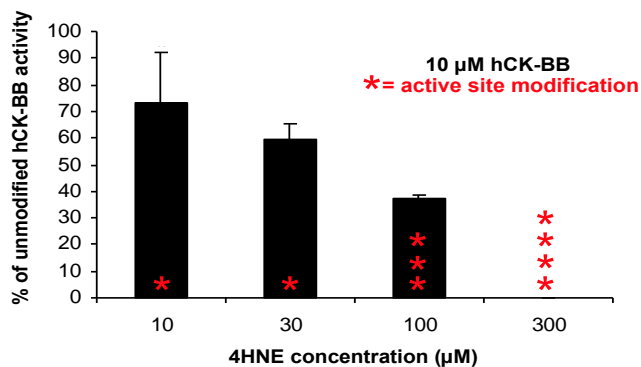
## Crystal structure of CK-BB showing the HNE adducts at active-site and non-active-site residues detected at 30 $\mu\text{M}$ 4HNE.



(crystal structure adapted from Eder et al., 1999)

HelenKim/UAB/Pharm700 (Eliuk et al., *Chem. Res. Toxicol.*, 2007)

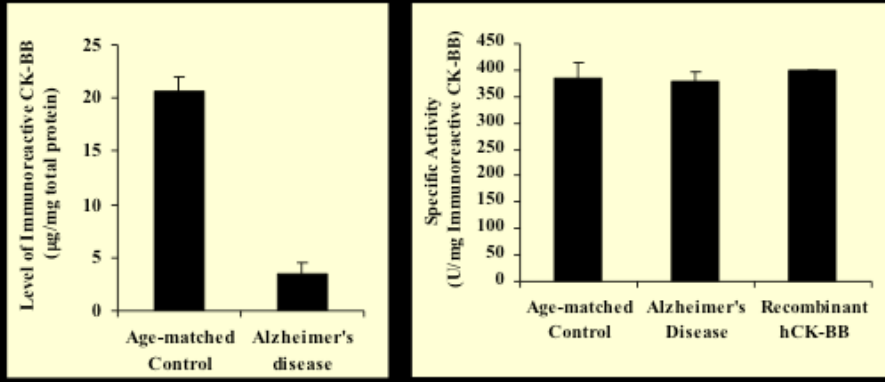
## Reduction of CK-BB activity correlated with increased HNE-adducts formed at active-site residues



(Eliuk et al., *Chem. Res. Toxicol.*, 2007)

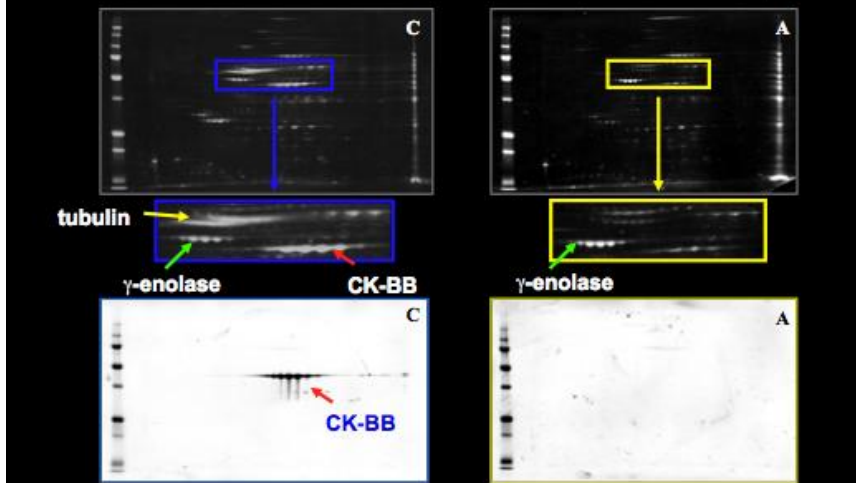


## Specific Activity of CK-BB in AD or CT cytosol was not different

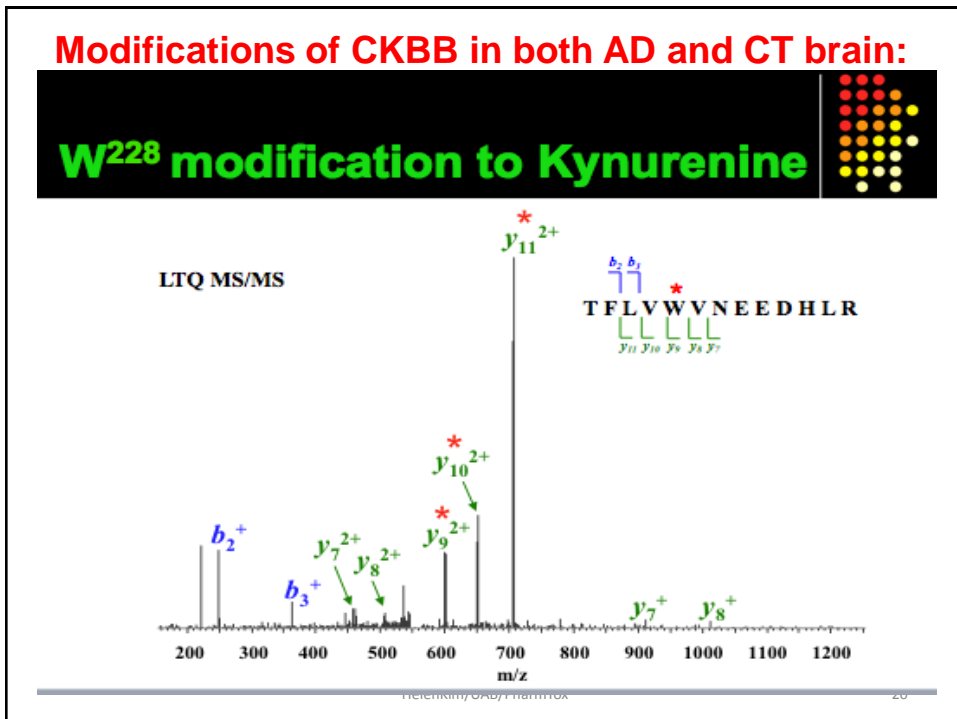
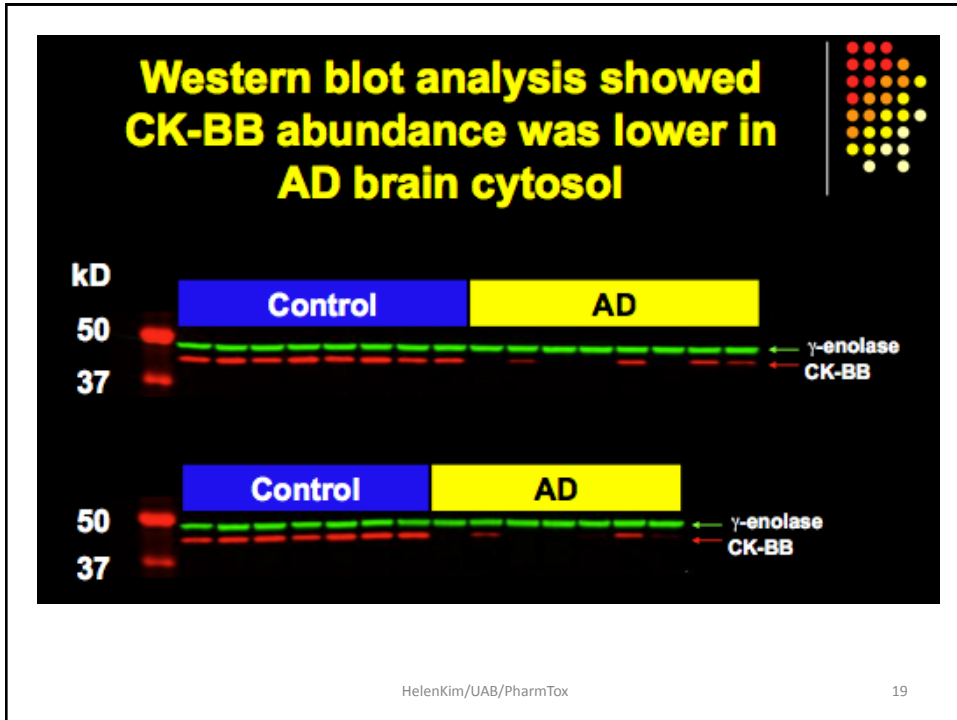


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## 2D-gels showed decreased CK-BB in AD cytosol fractions

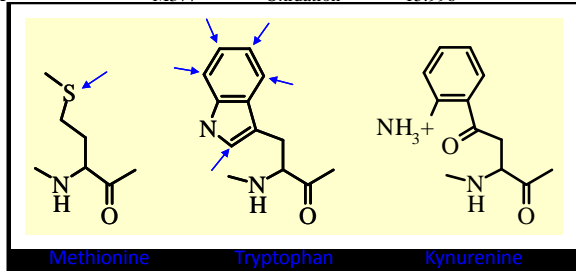


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## Modifications on CKBB detected by MS/MS analysis

Peptide Sequence	Modified Amino Acid	Modification	Mass Shift	AD	Age-matched Control
FPAEDEFPDLSAHNNHMAK	M30	Oxidation	15.998	✓	✓
FPAEDEFPDLSAHNNHMAK	M30	Di-oxidation	32.001	✓*	✓*
GIWHNDNK	W218	Oxidation	15.980	✓	✓
<b>TFLVWVNEEDHLR</b>	<b>W228</b>	<b>Kynurenine</b>	<b>3.999</b>	<b>✓*</b>	<b>✓**</b>
TFLVWVNEEDHLR	W228	Oxidation	16.005	✓	✓
TFLVWVNEEDHLR	W228	Di-oxidation	32.004	✓	✓
SKDYEFMWNPH	M272	Oxidation	15.997	✓	✓
LGFEVELVQMVDGVK	M352	Oxidation	15.990	✓	✓
LLIEMEQR	M363	Oxidation	15.995	✓	✓
LEOGOAIDDLMPAOK	M377	Oxidation	15.996	✓	✓



Structures courtesy of D. Stella

## Conclusions regarding the grapeseed studies

- **GSE has pleiotropic effects in the brain:**
  - gene expression/protein turnover;
  - protein oxidations;
  - These actions may be consistent with neuroprotection, since the majority are in the opposite direction to changes affecting these proteins in AD or models of dementia.
  - These were the first studies to identify specific proteins affected by dietary intake of a complex botanical mixture.

## **Conclusions from the studies with CKBB**

- **Incubation with HNE stoichiometrically inhibited hCKBB activity.**
  - **this was correlated with increased HNE adducts on CKBB, revealed by FT-ICR-MS.**
- **At the lowest activity, all four active-site residues of CKBB were HNE-modified.**
- **Thus, a combination of state of the art mass spectrometry and conventional biochemistry was optimal in determining the role of HNE adducts on CKBB function.**

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## **More conclusions from studies of CKBB**

- **In AD brain, CKBB was not detectably modified with HNE;**
- **Rather, oxidative modifications on CKBB were similar between AD and CT brains.**
  - **These non-differences correlated with a lack of difference in specific activity of CKBB between AD and CT.**

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