Using Molecular Methodology to Investigate the Mechanisms of Toxicity

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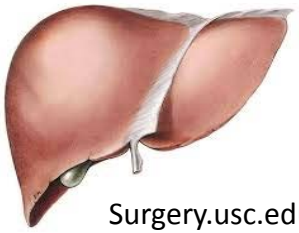
January 21, 2016
CalEPA - DPR

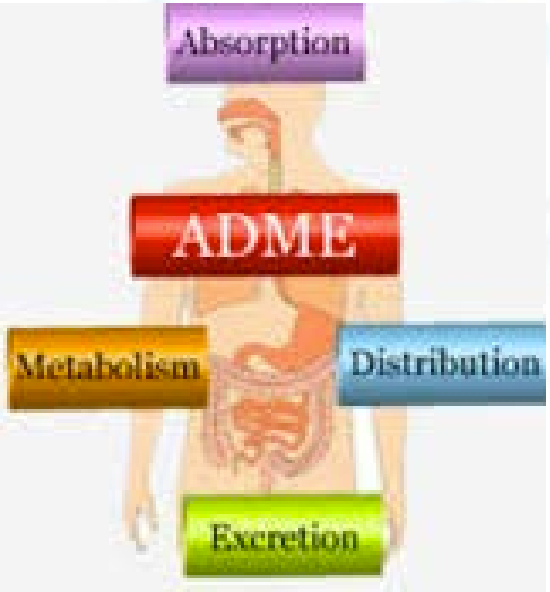
NIST
National Institute of Standards and Technology
U.S. Department of Commerce

MATERIAL MEASUREMENT LABORATORY

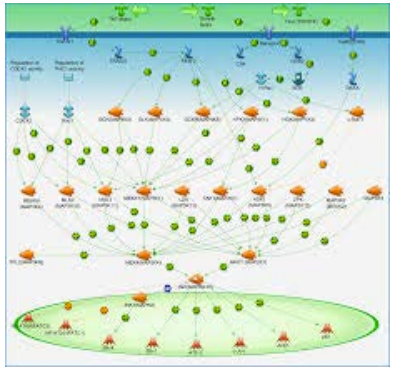
MOLECULAR TOXICOLOGY



Surgery.usc.edu



Roche.com

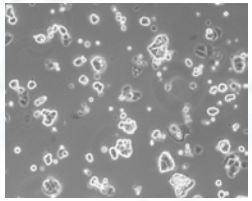


Bio-rad.com

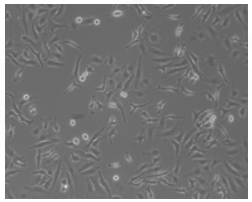
OUTLINE

1. Postdoctoral Research (National Institute of Standards and Technology (NIST), Dr. Miral Dizdaroglu)
    A. DNA damage analysis in human cells
    B. DNA extraction method development and DNA damage analysis in *C. elegans*
2. Graduate Work – *Daphnia magna* (U.C. Berkeley, Vulpe Laboratory)
    A. Chemical flame-retardants
    B. Silver nanowires

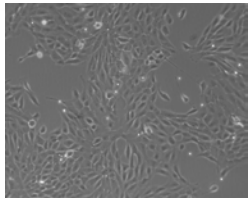
DNA DAMAGE IN HUMAN CELLS



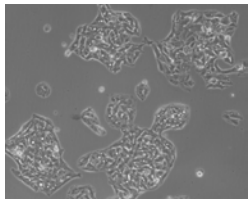
MCF7 – mammary gland epithelial metastatic adenocarcinoma



MCF10A – mammary gland epithelial non-cancer (fibrocystic tissue)



HeLa – cervical epithelial adenocarcinoma

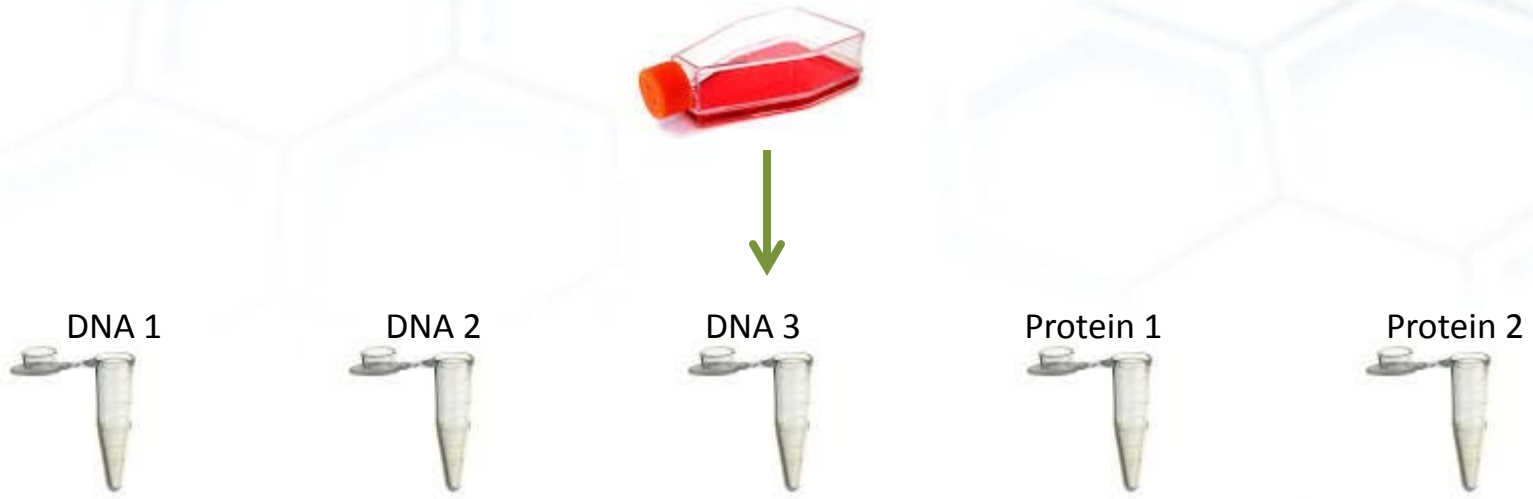


HepG2 – liver epithelia, hepatocellular carcinoma

Image credit: Alex Tona

EXPERIMENTAL PLAN

1. Grow cells as recommended
2. Aliquot cultures into multiple samples, 6 x 10^6 cells per sample.
3. Process “paired” samples.



DNA:

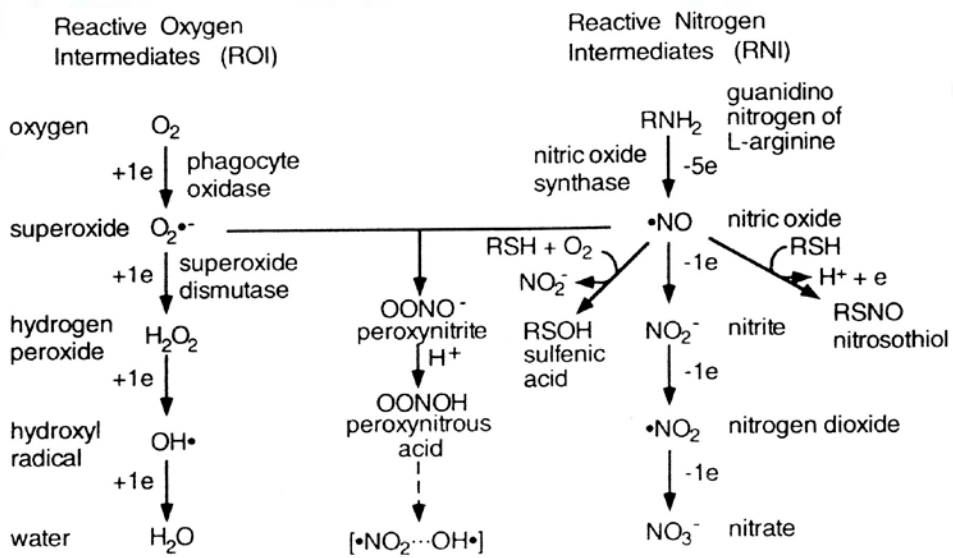
1. GC-MS/MS: *E. coli* Fpg/NTH proteins (5-OH-Cyt, 5-OH-Ura, thymine glycol, 5-OH-5-MeHyd, 8-OH-Gua,FapyGua, FapyAde, 8-OH-Ade)
2. LC-MS/MS: Nuclease P1, Phosphodiesterase I, Alkaline Phosphatase (8-OH-dGuo, 8-OH-dAdo, R-cdAdo, S-cdAdo)
3. GC-MS/MS: Nuclease P1, Phosphodiesterase I, Alkaline Phosphatase (R-cdGuo, S-cdGuo)

Protein

1. Total protein
2. Cytoplasmic and nuclear fractions

OXIDATIVE STRESS

- Oxidative stress is caused by many factors including chemical exposure and metabolism.
- Hydroxyl radical is most reactive species.
- Increased damage induces up-regulation of DNA repair enzymes



WORK-FLOW – ISOTOPE DILUTION

	DNA		Protein
Step 1 **Addition of Standards** **Hydrolysis**	**Enzymatic (Nuclease P1, phosphodiesterase I, alkaline phosphatase)**	**Enzymatic (DNA glycosylase)**	**Enzymatic (Trypsin)**
	all free modified and intact nucleosides	free modified bases (enzyme specific)	Tryptic peptides
Step 2 **Derivatization**	↓ ✕ ↓	↓ **Pyridine & BSTFA** ↓	↓ ✕ ↓
Step 3 **Analysis**	**LC-MS/MS**	**GC-MS/MS**	**LC-MS/MS**

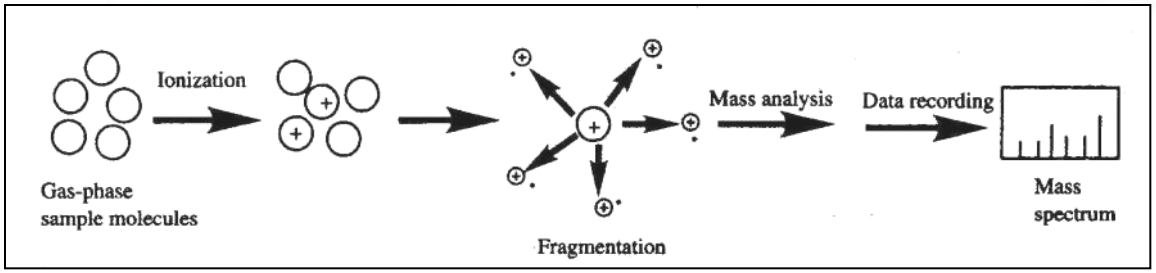
DNA:

1. GC-MS/MS: *E. coli* Fpg/NTH proteins (5-OH-Cyt, 5-OH-Ura, thymine glycol, 5-OH-5-MeHyd, 8-OH-Gua,FapyGua, FapyAde, 8-OH-Ade)
2. LC-MS/MS: Nuclease P1, Phosphodiesterase I, Alkaline Phosphatase (8-OH-dGuo, 8-OH-dAdo, R-cdAdo, S-cdAdo)
3. GC-MS/MS: Nuclease P1, Phosphodiesterase I, Alkaline Phosphatase (R-cdGuo, S-cdGuo)

GC-MS/MS AND LC-MS/MS

1. GC or LC separates a given mixture into its components.

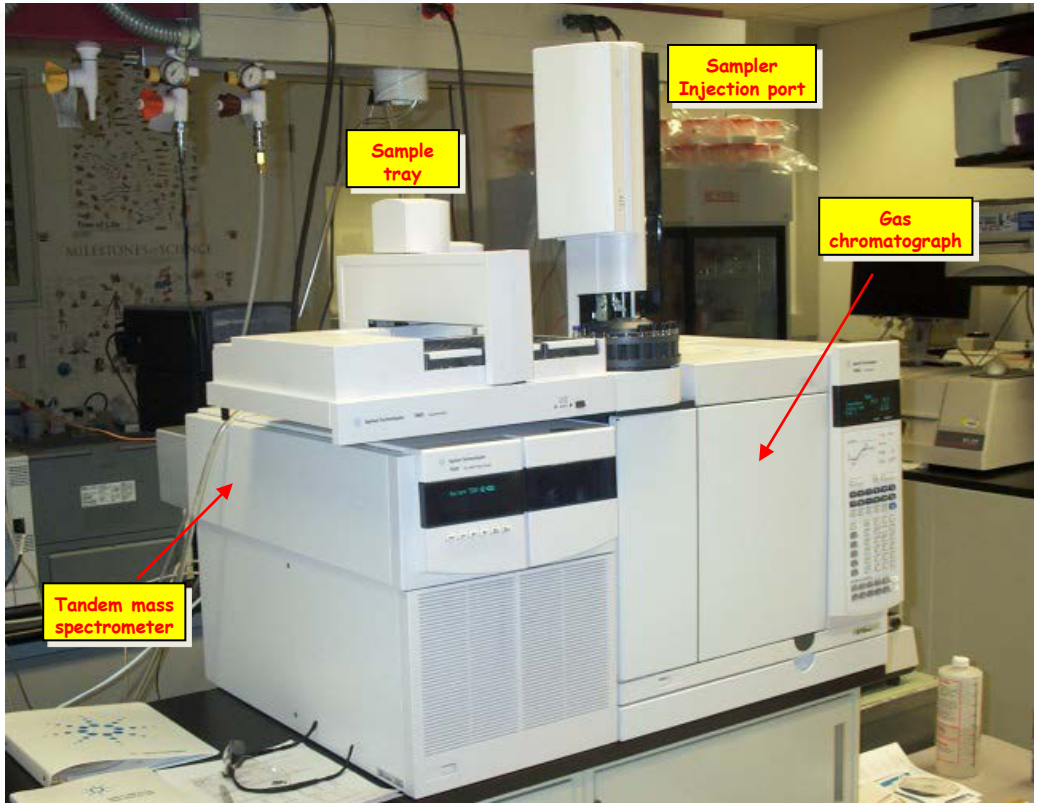
2. MS/MS identifies the components of the mixture separated by GC or LC, and quantifies them by isotope-dilution.

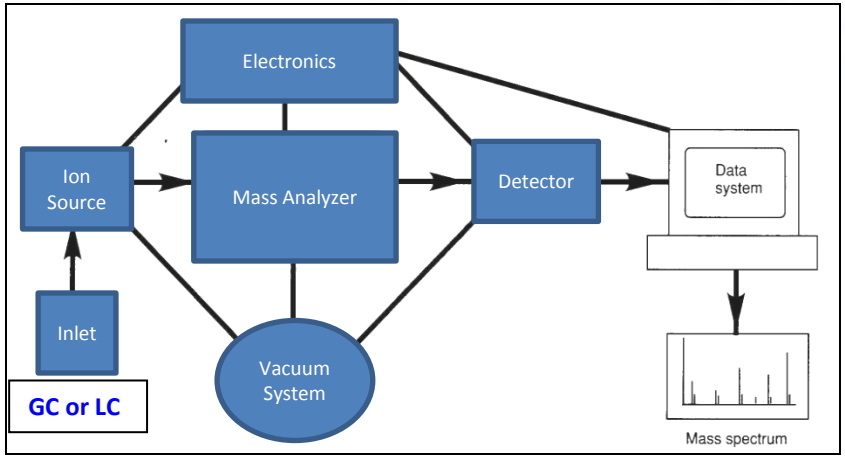


Methodology is specific, sensitive and quantitative

MASS-SPEC BASED TECHNIQUES

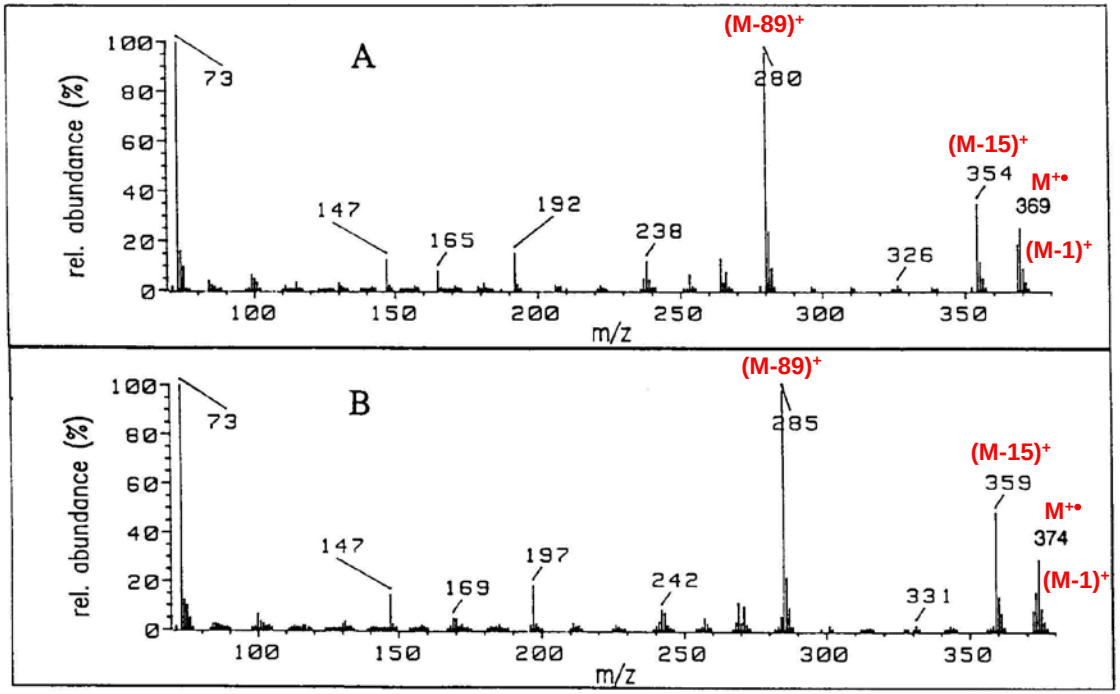
GC-MS/MS and LC-MS/MS – combination of two techniques

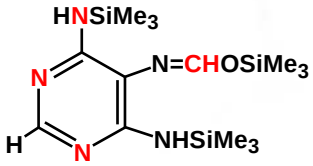




MASS SPECTRA OF TRIMETHYLSILYL DERIVATIVES

A: 4,6-diamino-5-formamidopyrimidine (FapyAde)



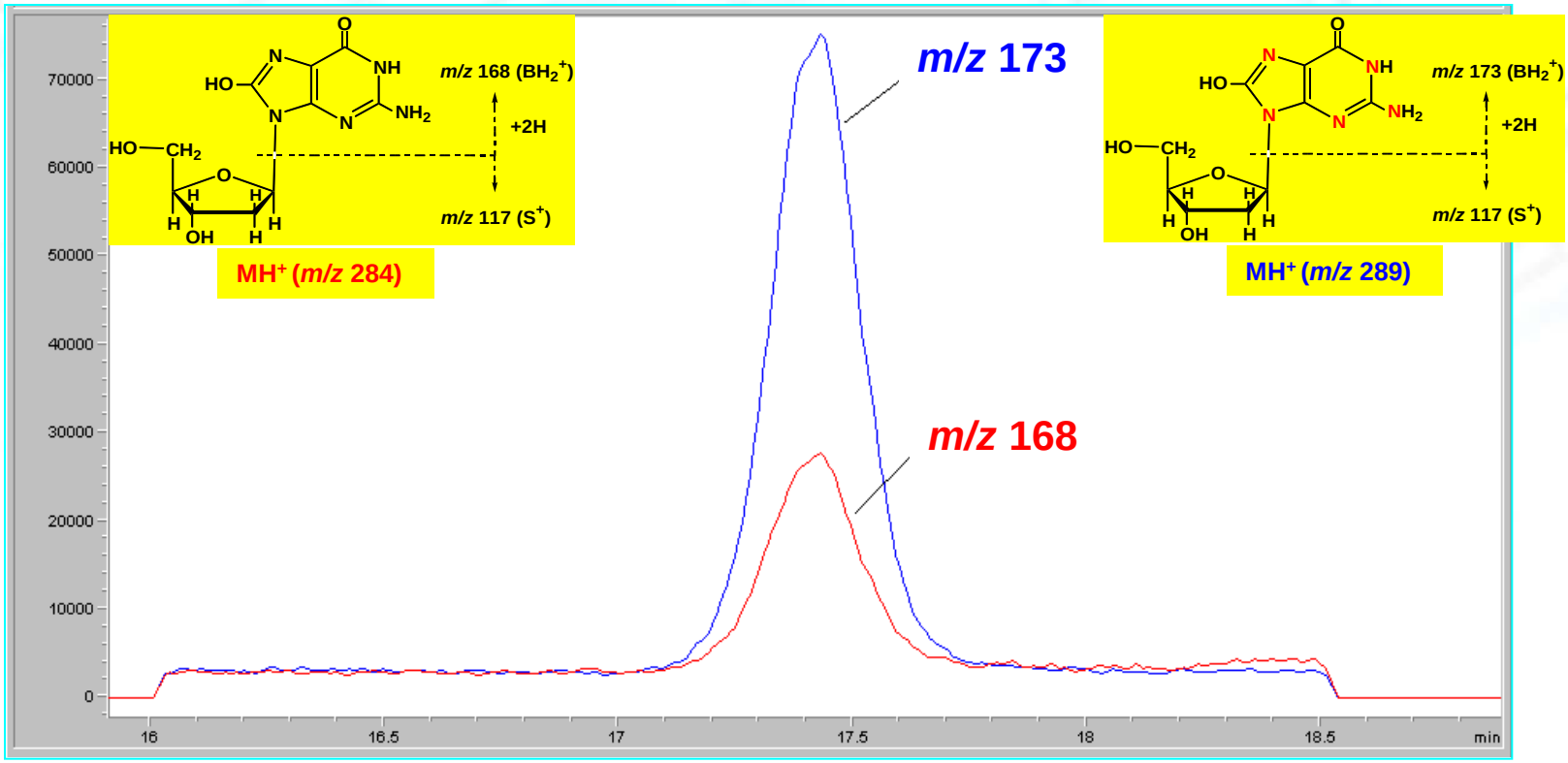


B: 4,6-diamino-5-formamidopyrimidine-$^{15}N_3$,^{13}C,^{2}H

Isotope-dilution

Dizdaroglu, M., *FEBS Lett.*. 315, 1-6, 1993

ION SIGNALS – STANDARD

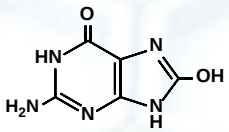


m/z 168: 8-hydroxy-2'-deoxyguanosine
m/z 173: 8-hydroxy-2'-deoxyguanosine-$^{15}N_5$

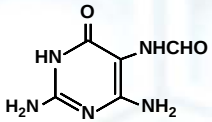
Dizdaroglu, M. , Jaruga, P. and Rodriguez, H., *Nucleic Acids Res.* 29, *e12*, 2001

OXIDATIVELY INDUCED DNA BASE LESIONS

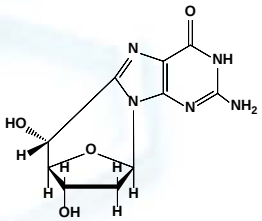
guanine-derived products



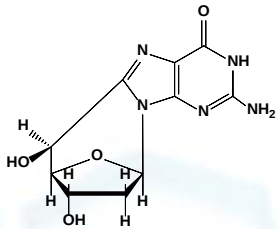
8-hydroxyguanine



2,6-diamino-4-hydroxy-5-formamidopyrimidine

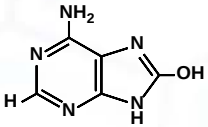


(5'*R*)-8,5'-cyclo-2'-deoxyguanosine

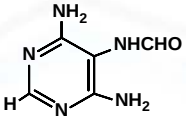


(5'*S*)-8,5'-cyclo-2'-deoxyguanosine

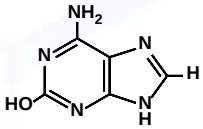
adenine-derived products



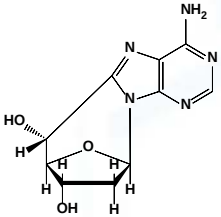
8-hydroxyadenine



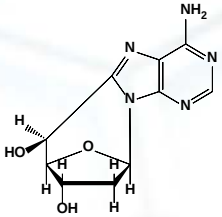
4,6-diamino-5-form-amidopyrimidine



2-hydroxyadenine

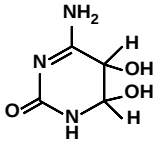


(5'*R*)-8,5'-cyclo-2'-deoxyadenosine

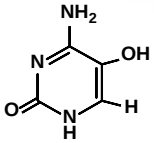


(5'*S*)-8,5'-cyclo-2'-deoxyadenosine

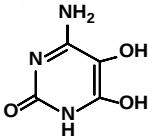
cytosine-derived products



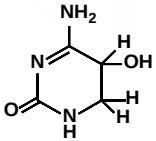
cytosine glycol



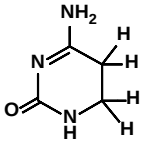
5-hydroxycytosine



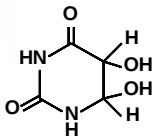
5,6-dihydroxy-cytosine



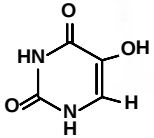
5-hydroxy-6-hydrocytosine



5,6-dihydrocytosine

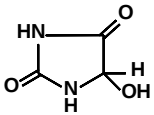


uracil glycol

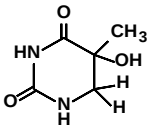


5-hydroxyuracil

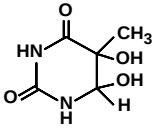
thymine-derived products



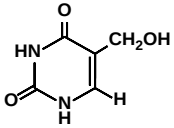
5-hydroxy-hydantoin



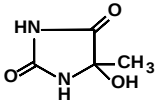
5-hydroxy-6-hydro-thymine



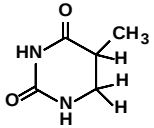
thymine glycol



5-(hydroxymethyl)-uracil



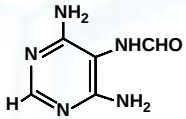
5-hydroxy-5-methylhydantoin



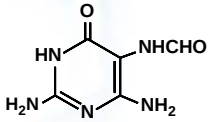
5,6-dihydrothymine

Reviewed in: Dizdaroglu, M. and Jaruga, P., *Free Radic. Res.* 46, 382-419, 2012

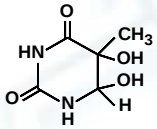
DNA LESION LEVELS IN HUMAN CELLS



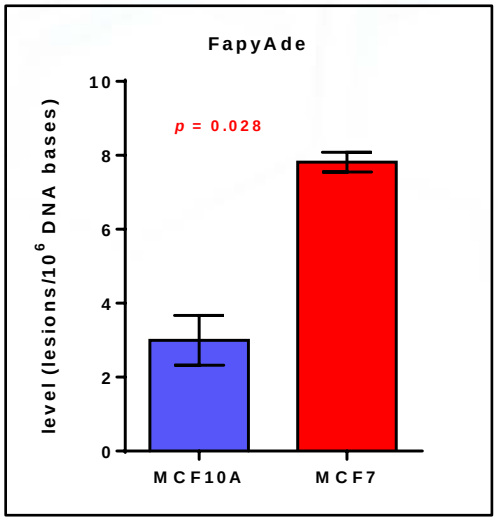
4,6-diamino-5-form-amidopyrimidine

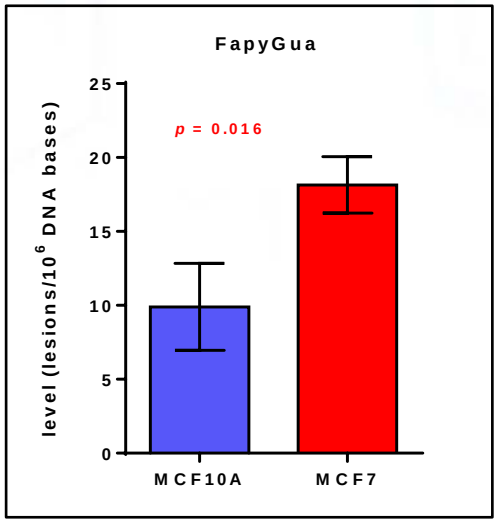


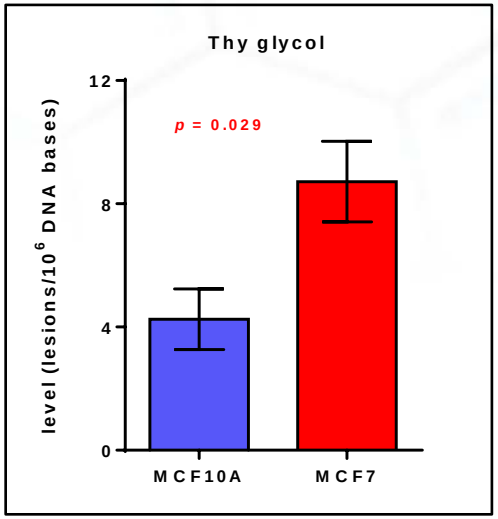
2,6-diamino-4-hydroxy-5-formamidopyrimidine



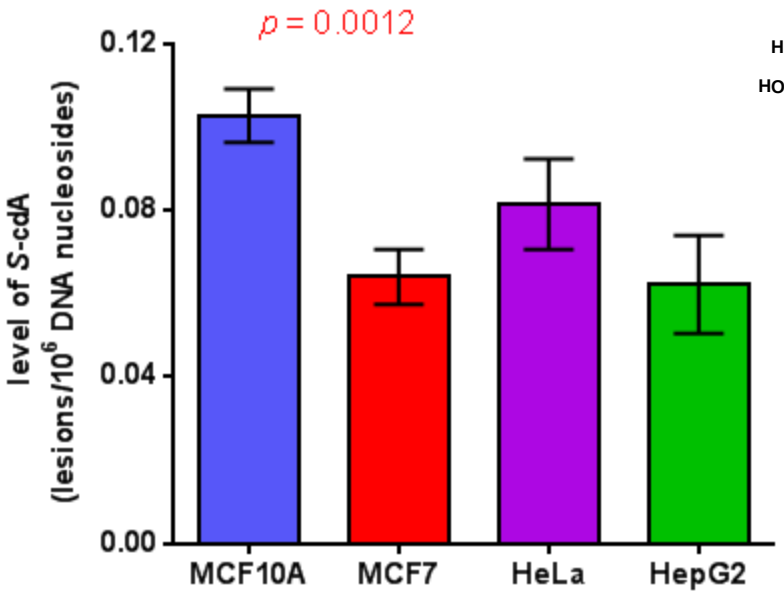
thymine glycol

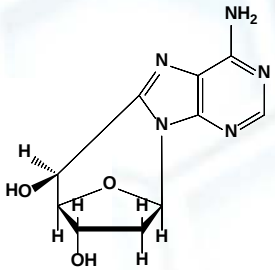






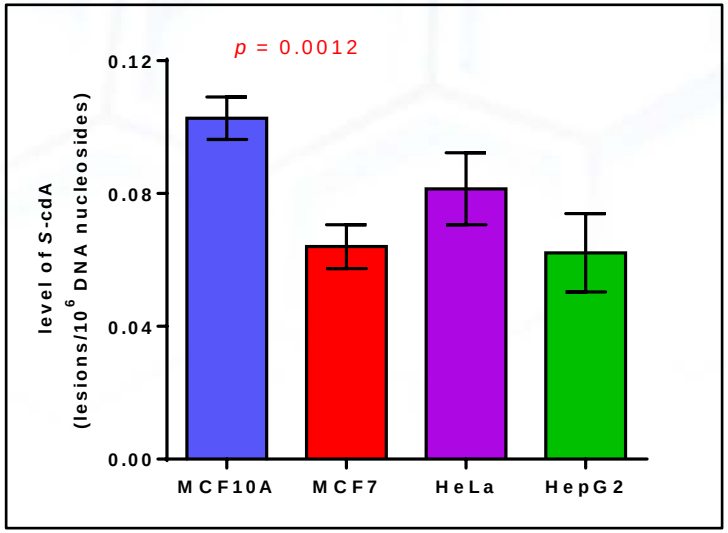
DNA LESION LEVELS IN HUMAN CELLS – CYCLO-DEOXYADENOSINE



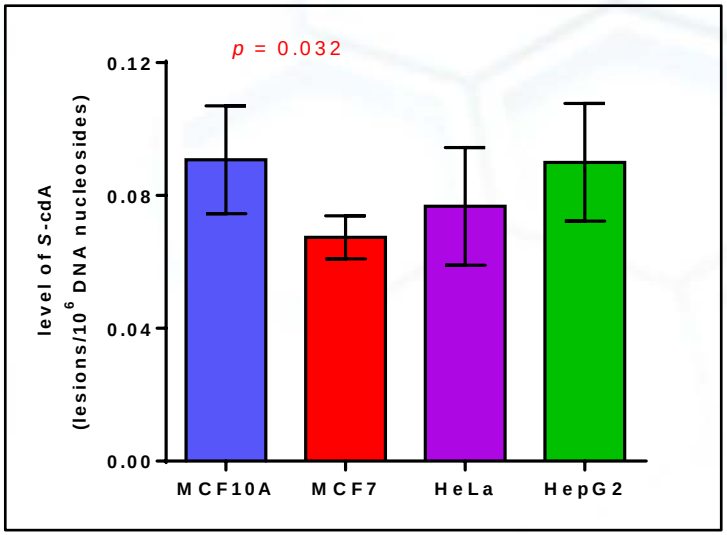


DNA LESION LEVELS IN HUMAN CELLS – CYCLO-DEOXYADENOSINE

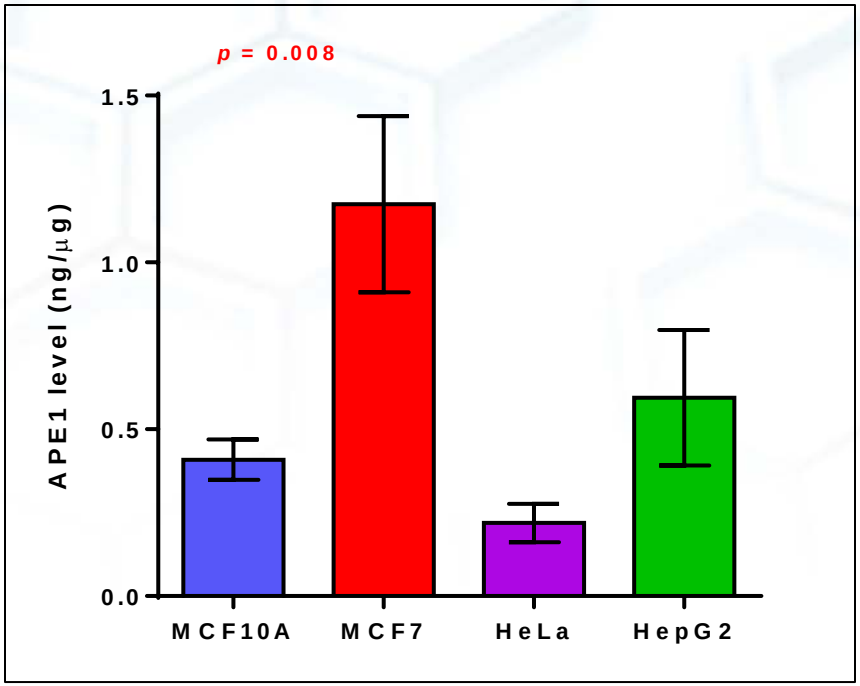
GC-MS/MS



LC-MS/MS



APE1 LEVELS IN HUMAN CELLS



p-values	MCF10A	MCF7	HeLa	HepG2
MCF10a	--	0.008	≤0.0001	ns
MCF7	--	--	≤0.0001	0.0006
HeLa	--	--	--	≤0.0001
HepG2	--	--	--	--

IN VITRO SUMMARY

- Preliminary analysis shows that lesions are detected at different levels in mammary cancer versus non cancer cells.
    - Lesions repaired by BER are found at higher levels in cancer cells.
    - Lesions repaired by NER are found at higher levels in normal cells.
- The non-cancerous cell line (MCF10a) also has a lower level of DNA repair enzyme APE1 than the corresponding cancerous cell (MCF7).

IN VITRO NEXT STEPS

- Incorporate additional DNA replicates, MTH1 data
- Colleagues at NIST may investigate effects of specific toxins, such as cigarette smoke or environmental contaminants

DNA DAMAGE IN *CAENORHABDITIS ELEGANS*

a.k.a. *C. elegans,* 'worms,' nematodes

Usually lives in soil

Used for medical and environmental studies

Photo credit: Sanem Hosbas Coskun

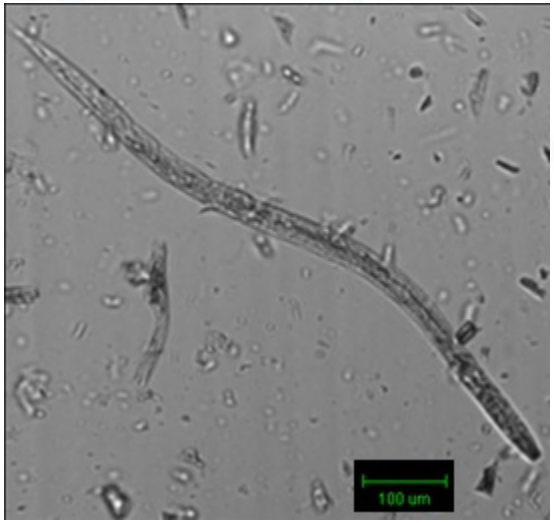


Photo credit: Mike Carrier

MOLECULAR STUDIES WITH THE NEMATODE

1. Microarrays, blots, plate assay kits
2. RNAi
3. Transgenic/ genetic
4. DNA damage – qPCR
5. Apoptosis
6. Cell cycle

NEMATODE GOALS

1. Grow healthy populations of nematodes in liquid growth media (S-basal, CeHR)
2. *In silico* investigate protein homology to DNA repair enzyme with NIST standards
3. Develop high-salt DNA extraction method for *C. elegans*
4. Extract DNA from nematodes with high-salt and phenol-based protocols
5. Measure background DNA lesion levels
6. Determine if one growth medium or extraction type induces more DNA lesions.
7. Conduct toxicity studies with commercially relevant nanomaterials)

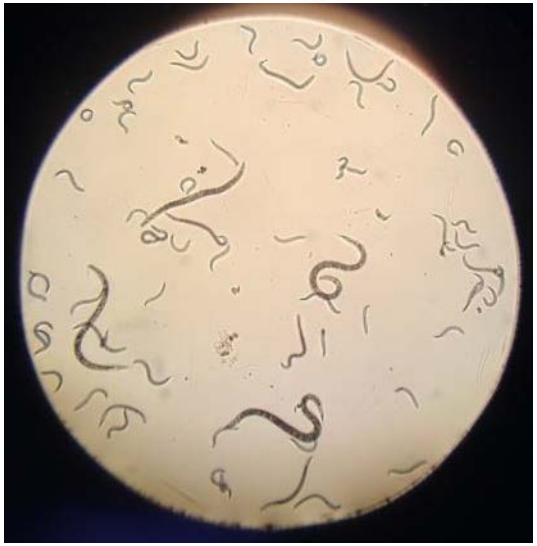
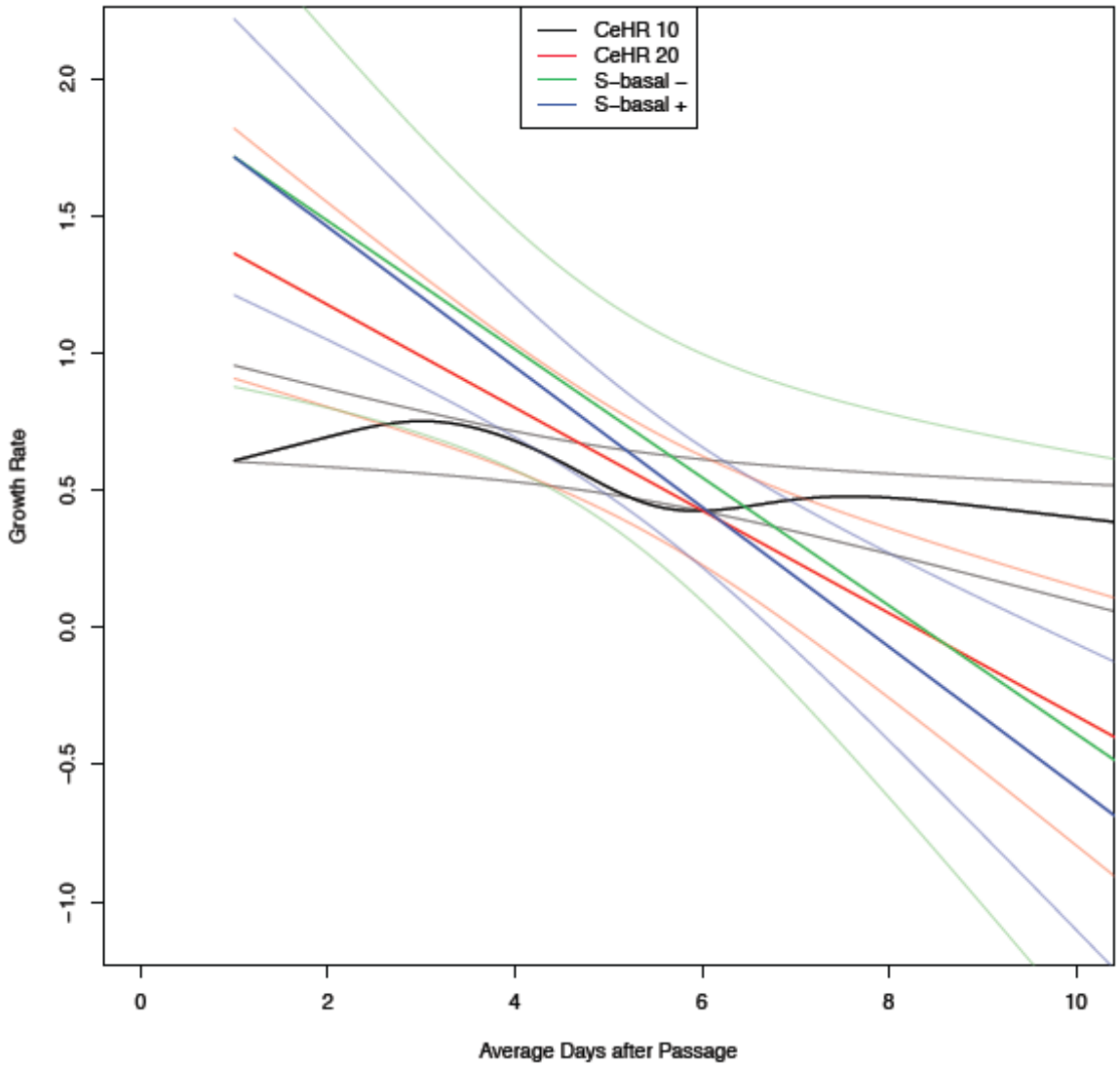


Photo credits: Sanem Hosbas Coskun

WORM GROWTH


CeHR 10
CeHR 20
S–basal –
S–basal +
Growth Rate
2.0
1.5
1.0
0.5
0.0
−0.5
−1.0
0
2
4
6
8
10
Average Days after Passage

DNA REPAIR ENZYME HOMOLOGY

DNA repair enzymes: APE1, OGG1, MTH1, NTH1, Pol-β, NEIL1

- Searched *C. elegans* proteome for DNA repair enzymes
- 7 (out of 27,164) proteins were somewhat homologous to human (e.g., NTH1 - DNA glycosylase)
- Used PeptideCutter – ExPASy to find computationally "digest" *C. elegans* proteins into peptides
- Resulting tryptic peptides of *C. elegans* were different from human peptides.

C. elegans DNA repair enzymes cannot be quantified using our standards –

We can not (yet) quantify DNA repair enzymes in _C. elegans_

C. elegans genome: Science. 1998 Dec 11;282(5396):2012-8.

HIGH-SALT DNA EXTRACTION

Grow nematodes, divide into aliquots of ~500,000 nematodes, freeze at -80 ° C

Incubate with 2 mg/ mL Proteinase K, 55 °C for 2 hr → Crash out proteins with saturated NaCl → Centrifuge at 15,000 x g for phase separation

Precipitate DNA out of supernatant with EtOH → Treat with RNase, clean up

QUALITY AND QUANTITY OF DNA

Quality:

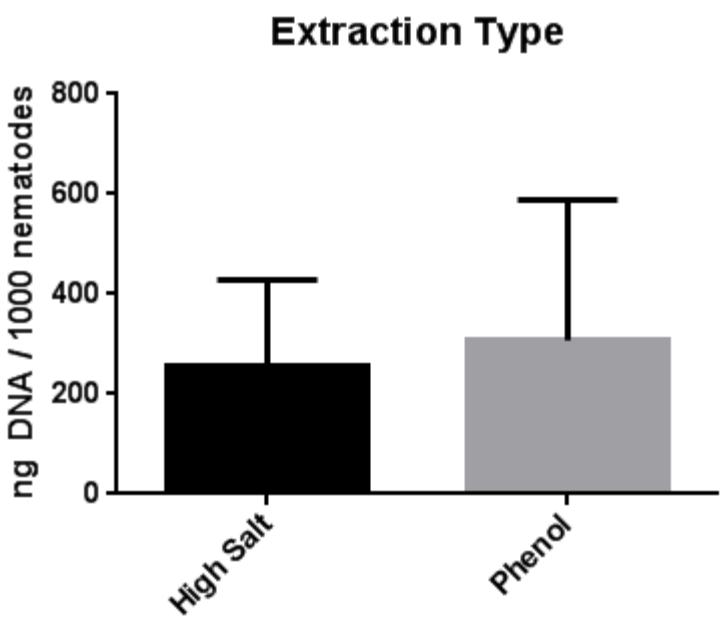
- 260/280 photospectrometer readings
- Shape of spec trace
- Percent RNA (qubit)
- Amount DNA (coomassie blue/ Bradford assay)
- Size of fragments (pulse-field gel)
- Source of DNA (E. coli, bovine or C. elegans, dPCR)

Quantity

- Spectrophotometry
- Qubit (not as reliable!)
- NIST DNA standard

HIGH-SALT VERSUS PHENOL DNA EXTRACTION

DNA yields are not significantly different (two-tailed t-test, p-value = 0.506)



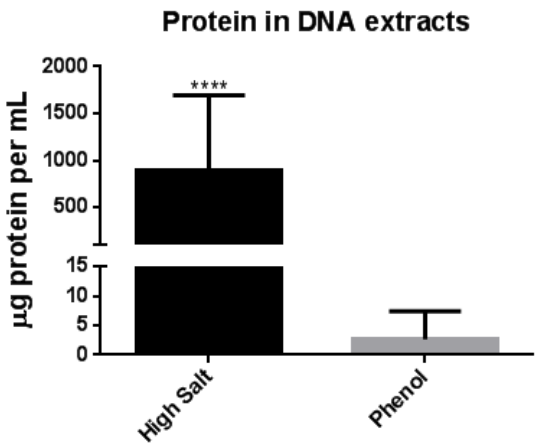
Extraction	N
HS	19
phenol	18

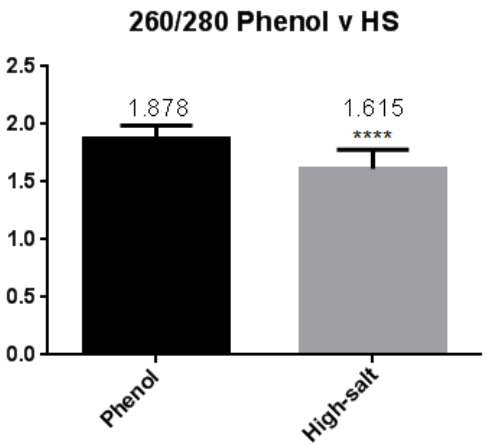
PROTEIN IN DNA EXTRACTS

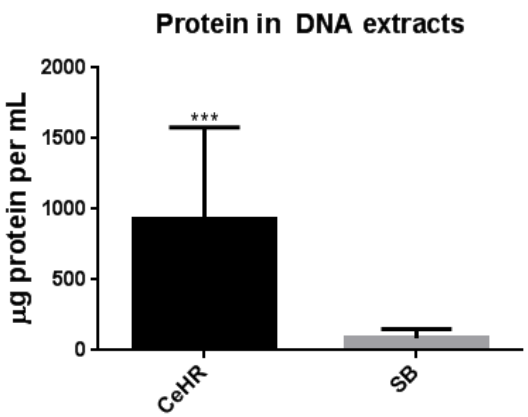
All error bars are SD.
*** $p < 0.001$
**** $p < 0.0001$

Sample	N
HS	18
Phenol	19
S-basal	10
CeHR	24

High-salt extracts contain more proteins than phenol extracts.
Extracts from S-basal cultures contain less proteins than those form CeHR cultures







PRELIMINARY LESION ANALYSIS IN *C. ELEGANS*

High-salt extracts from worms grown in S-basal.
Phenol extraction caused increased background DNA damage levels (not shown)

lesions/ 10^6 bases	FapyAde	FapyGua	8-OH-Gua	5-OH-5MeHyd	*R*-cdA	*S*-cdA	*R*-cdG	*S*-cdG
average	7.06	15.38	17.73	28.49	0.30	0.13	13.10	22.90
sd	1.72	4.04	8.26	10.57	0.05	0.01	0.40	0.84

C. ELEGANS SUMMARY

- Used numerous molecular techniques to extract DNA and measure it's quantity and quality as well as measure levels of DNA lesions.
- Next steps for NIST Nanomaterial Genotoxicity group is to conduct toxicity assays, using standardized culture and DNA extraction methods and other molecular methodologies.
  - spICP-MS, fluorescent plate assays, etc.

THANK YOU!

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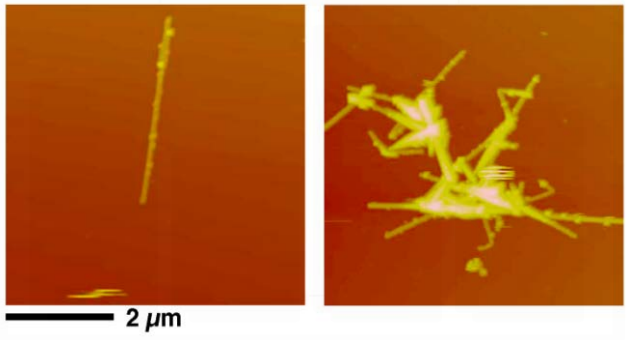
Monique Johnson
Jamie Almeida
David Catoe
Alex Tona
Prasad Reddy
Mike Carrier

645.00
Mike Tarlov
Carole Young

Part 2: *Daphnia magna*
5 μm

Silver Nanowires

- Anisotropic
- Increasingly used in nano-scale devices and pharmaceuticals: emerging concern



Atomic Force Microscopy of short PVP AgNWs

Two lengths:

- Short (S) 30 nm x 2um
- Long (L) 65 nm x 20 um

Two coatings:

- Silica (SiO_2)
- Polyvinyl pyrolidinone (PVP)

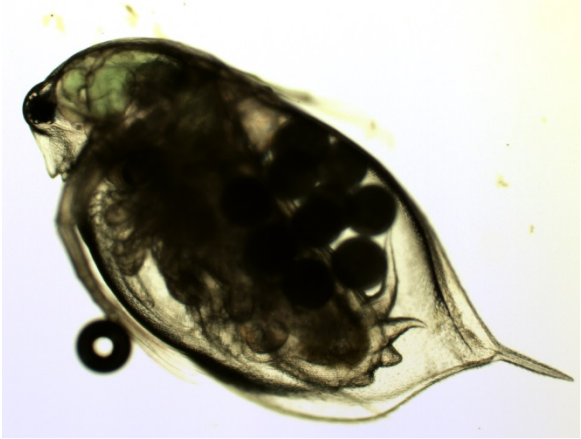
Aquatic Toxicity of Silver

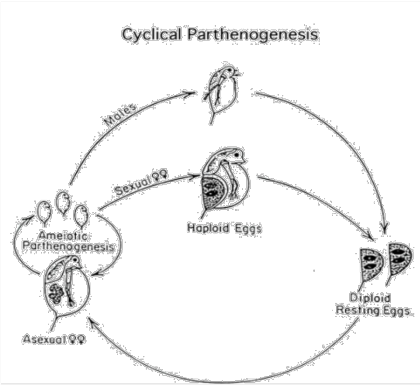
- Competes for Na^+ uptake at gill
- Binds Mg^{2+}-binding site cysteine on the Na^+/K^+ ATPase
  - Double whammy to ionoregulation
- General stress response, increased ammonia, fluid volume changes, and hemoconcentration

(1) Péqueux A. Journal of Crustacean Biology 1995;15(1):1-60.
(2) Andren A, Bober T editors. Society of Environmental Toxicology and Chemistry;2002.
(3) Bianchini A., Wood CM. Environ Toxicol Chem 2003 Jun;22(6):1361-1367.

Our Questions:

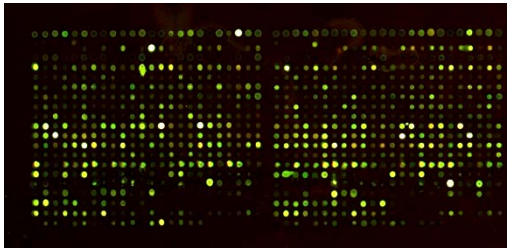
- Is it the Ag^+, or is it the particle?
- How do they cause toxicity?



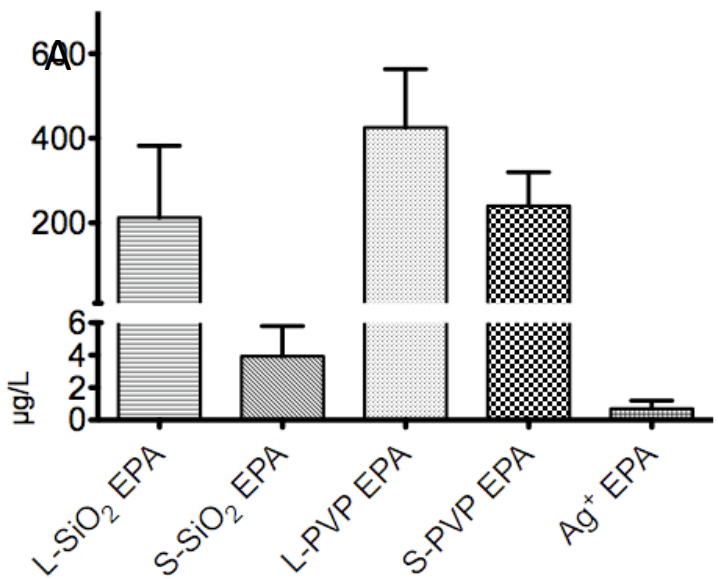


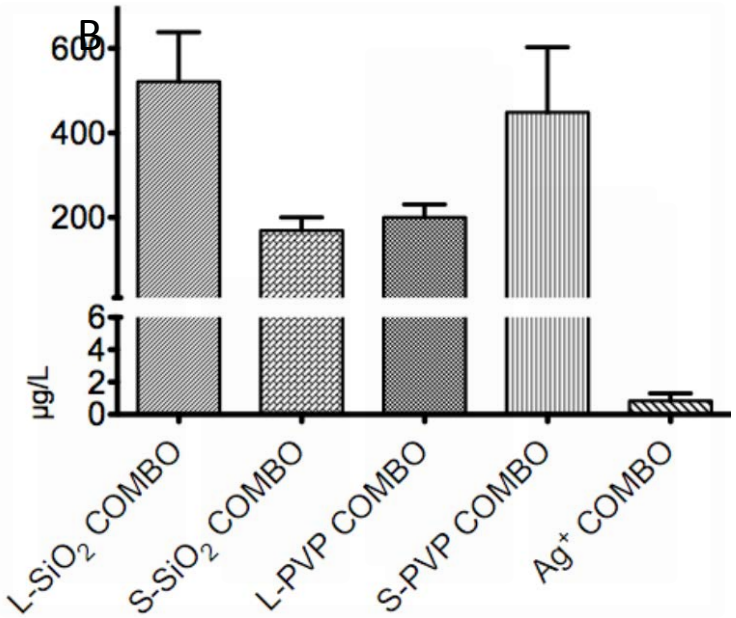
Assays

- Acute LC_{50}
- Gene expression analysis and pathway analysis
  - Microarray, qPCR
  - High-throughput sequencing
- Hemolymph extraction



Acute LC_{50} Results





Acute, 24-hour LC_{50}s for AgNW and Ag^+ on *Daphnia magna* in A) COMBO media and B) EPA media. LC_{50}s are measured in micrograms silver per liter.

Scanlan LD et al. *ACS Nano* **2013,** 7, 10681-10694. DOI: 10.1021/nn4034103

Ag^+ in Media

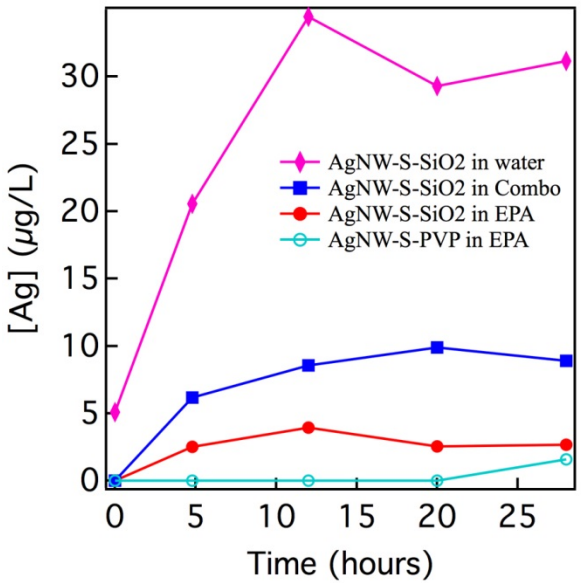
Measure silver release

Add 12 mg/L Ag NW to water, EPA, COMBO media

Take aliquots, filter through 0.02 µm filter

all filters retain some Ag^+

Measure total dissolved silver with ICP-MS



S – SiO_2 in water

S – SiO_2 in COMBO

S – SiO_2 in EPA

S – PVP

- SiO_2-coated NW most reactive!
- Silver release depends on media
- Conditions not on graph had no detectable Ag+ release

Is Toxicity Caused by Ag^+?

Does Ag oxidation *through* SiO_2 coating explain toxicity?

- Assume Ag^+ release in media proportional to Ag^0 concentration
- Scale Ag^+ release to LD_{50} values

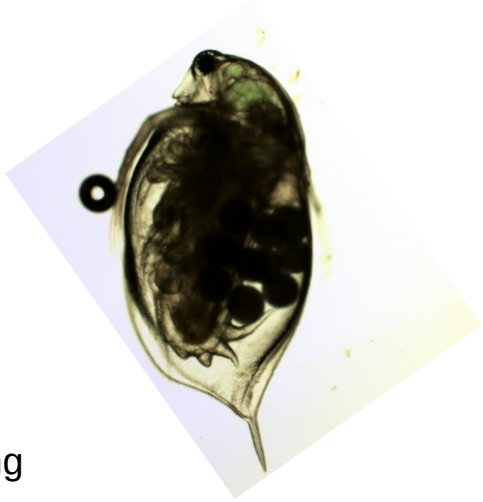
LD_{50}		Predicted $[Ag^+]$ at LD_{50}
S-SiO_2 in COMBO = 167 µg/L	…	$[Ag^+]$ = 0.14 µg/L
S-SiO_2 in EPA = 4 µg/L	…	$[Ag^+]$ = 0.001 µg/L
$AgNO_3$ ~ 0.8 µg/L		

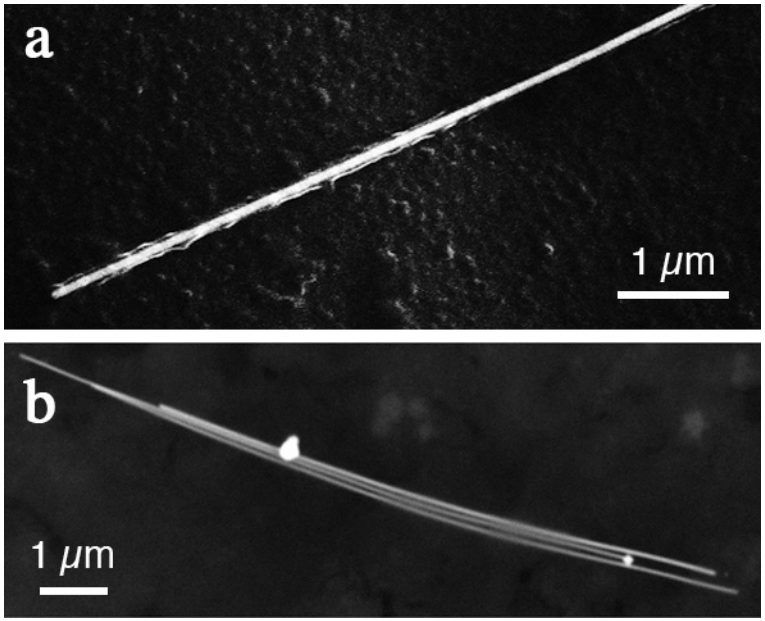
(Much) smaller values

Ingestion = Absorption?

SEM imaging of AgNW from *Daphnia* hemolymph

All types of nanowires were found in the hemolymph





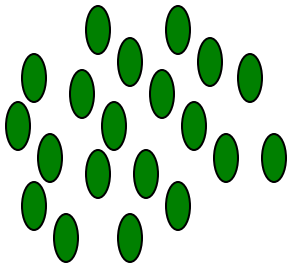
PVP-coated AgNW

Strange fibrous coating

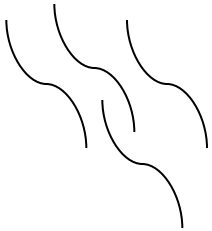
SiO_2-coated AgNW

Complete dissolution of coating
Possible recrystallization

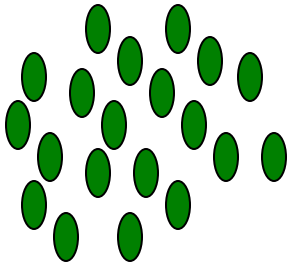
Gene Expression Assays



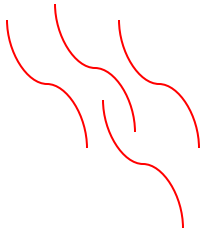
Exposed (1/10 LC_{50})



DGE



Control

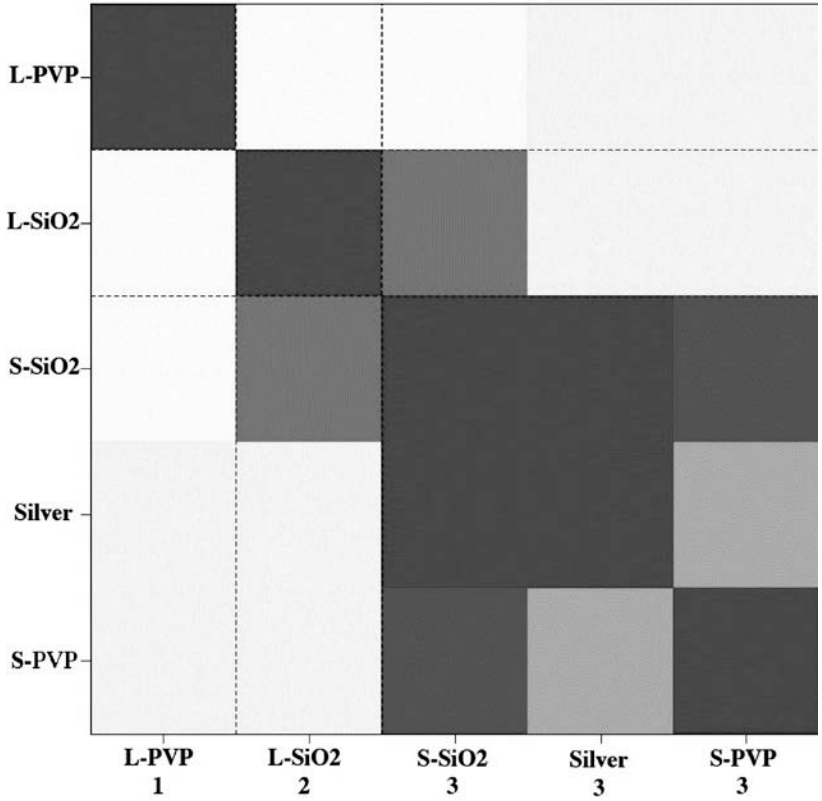


(confirmed with qPCR)

4 control x 4 treated = 16 comparison sets

Comparison of DGE Profiles

- HOPACH



Hierarchical ordered partitioning and collapsing hybrid (HOPACH) analysis of gene expression data from AgNW-exposed *versus* media-only control daphnids. HOPACH clustered data into three groups based on similarity of gene expression profile. Groups are labeled on the *x*-axis as 1 (L-PVP-NW), 2 (L-SiO2-NW), or 3 (S-SiO2-NW, Ag+, and S-PVP-NW).

KEGG Pathway Analysis

TABLE 3. KEGG Pathway Analysis of Gene Expression Data from Daphnia magna Exposed to AgNWs Identified the Enrichments of Different Biological Pathways[a]

affected biological pathway	L-PVP	L-SiO_2	S-PVP	silver
oxidative phosphorylation				5.3×10^{-04}
spliceosome	0.02			
peroxisome		0.02		
lysosome		0.04		
ribosome	0.01		0.01	1.8×10^{-4}
progesterone-mediated oocyte maturation		0.01		
metabolism of xenobiotics by cytochrome P450		0.02		
drug metabolism - cytochrome P450		0.03		
retinol metabolism		0.05		

[a] The *p* values 0.05 or less were considered significant. KEGG analysis on S-SiO_2-NW data found no statistically significant results.

Enrichment Analysis

TABLE 4. Blast2GO Analysis Shows Significant Enrichment of Functional Gene Groups PVP-NW and Ag^+ Exposures

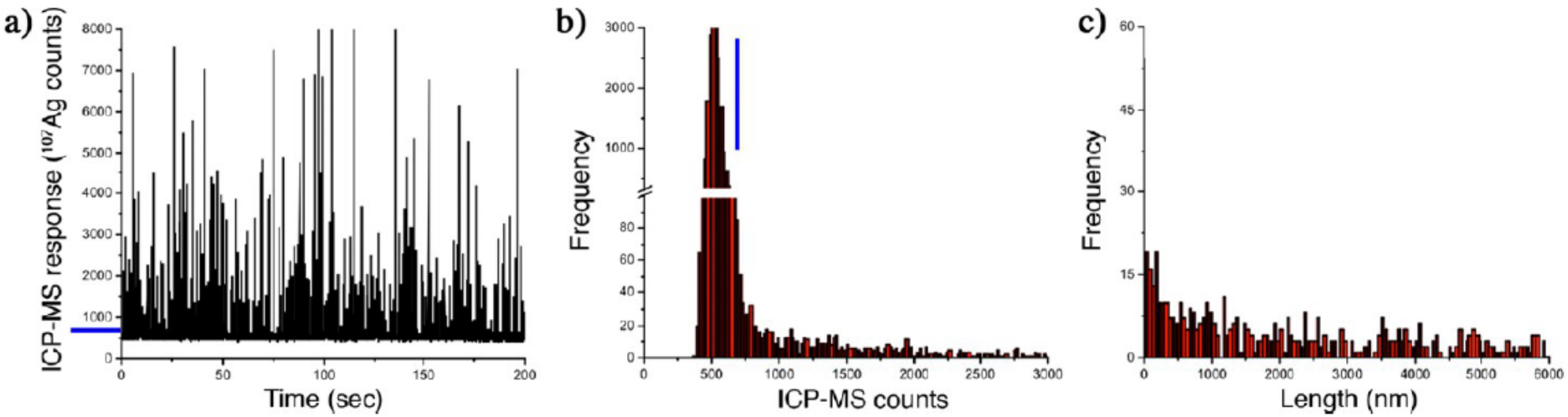
enriched molecular function[a]	exposure condition
structural constituent of cuticle (GO:0042302)	L-SiO_2, S-SiO_2
structural molecule activity (GO:005198)	L-SiO_2, silver
structural constituent of ribosome (GO:0003735)	silver
ribosome biogenesis (GO:0042254)	silver
ribosome (GO:0005840)	silver
ribonucleoprotein complex biogenesis (GO:0022613)	silver
cellular component biogenesis at cellular level (GO:0071843)	silver
ribonucleoprotein complex (GO:0030529)	silver
cytosolic ribosome (GO:0022626)	silver

[a] GO: term corresponds to the gene ontology identification in Blast2GO.

Mechanisms

- KEGG, B2G and HOPACH indicate diverse range of biological endpoints
- Toxicity appears to be attributed to both silver and to the silver nanowire

spICP-MS



- Quantified number and size of particles in media (growth medium, hemolymph)
- Measured accumulation of nanomaterials into *Daphnia* (wires and particles)
- Ionic silver exposure also resulted in accumulation of silver nanoparticles

Scanlan LD et al. *Environ. Sci. Technol.*, 2015, 49 (12), pp 7400–7410

Flame-retardants

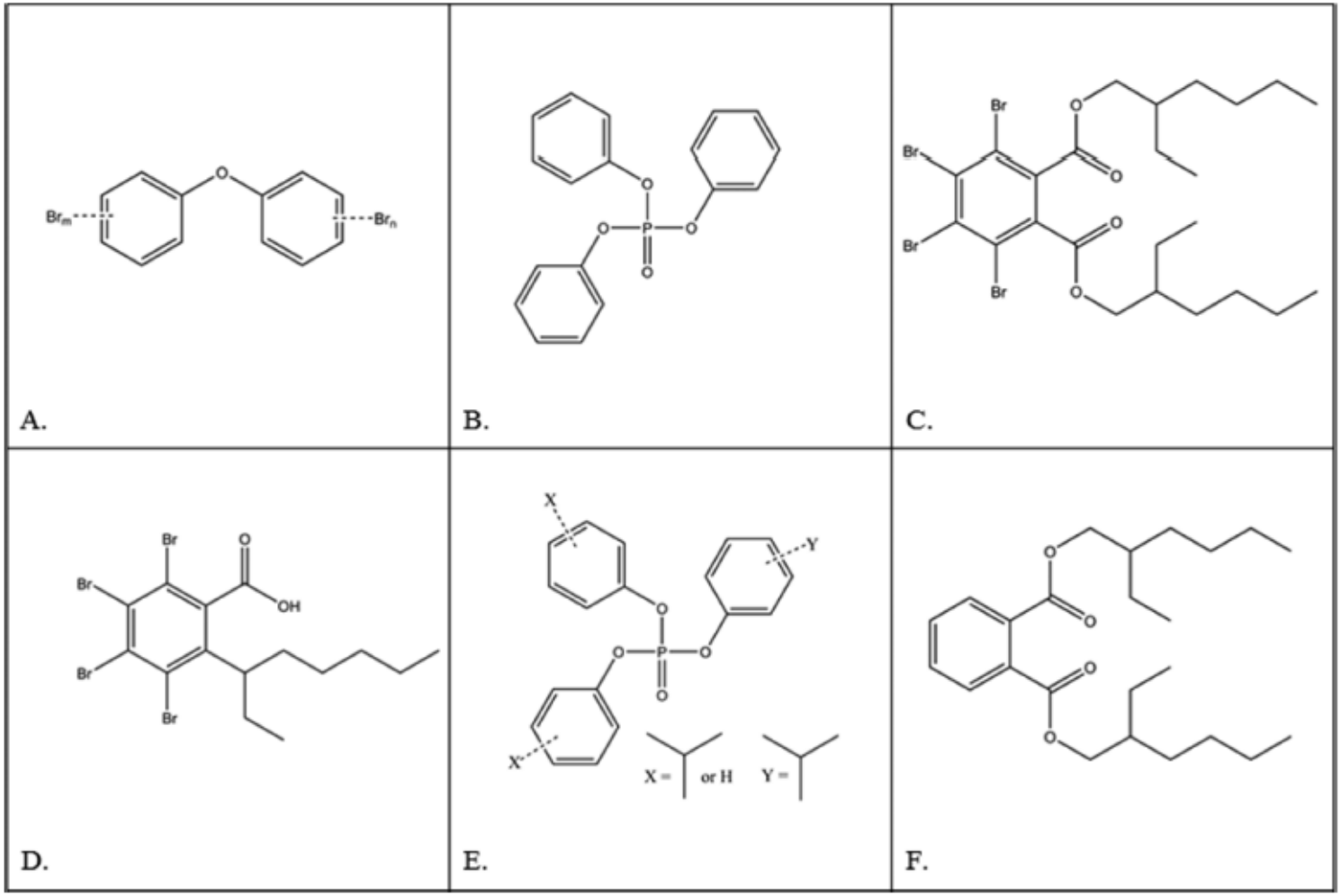


Figure 1. Molecular structures of chemical flame-retardants. A. Polybromodiphenyl ether. B. Triphenyl phosphate (TPhP). C. bis(2-ethylhexyl) tetrabromophthalate (BEH-TEBP). D. bis(2-ethylhexyl) tetrabromobenzoate (EH-TBB). E. Isopropylated triaryl phosphates (ITP). F. bis(2-ethylhexyl) phthalate (BEHP). B–E are components of Firemaster 550. C and D are components of Firemaster BZ-54. E contains a mixture of molecules with one, two or three isopropyl-substituted phenol rings.

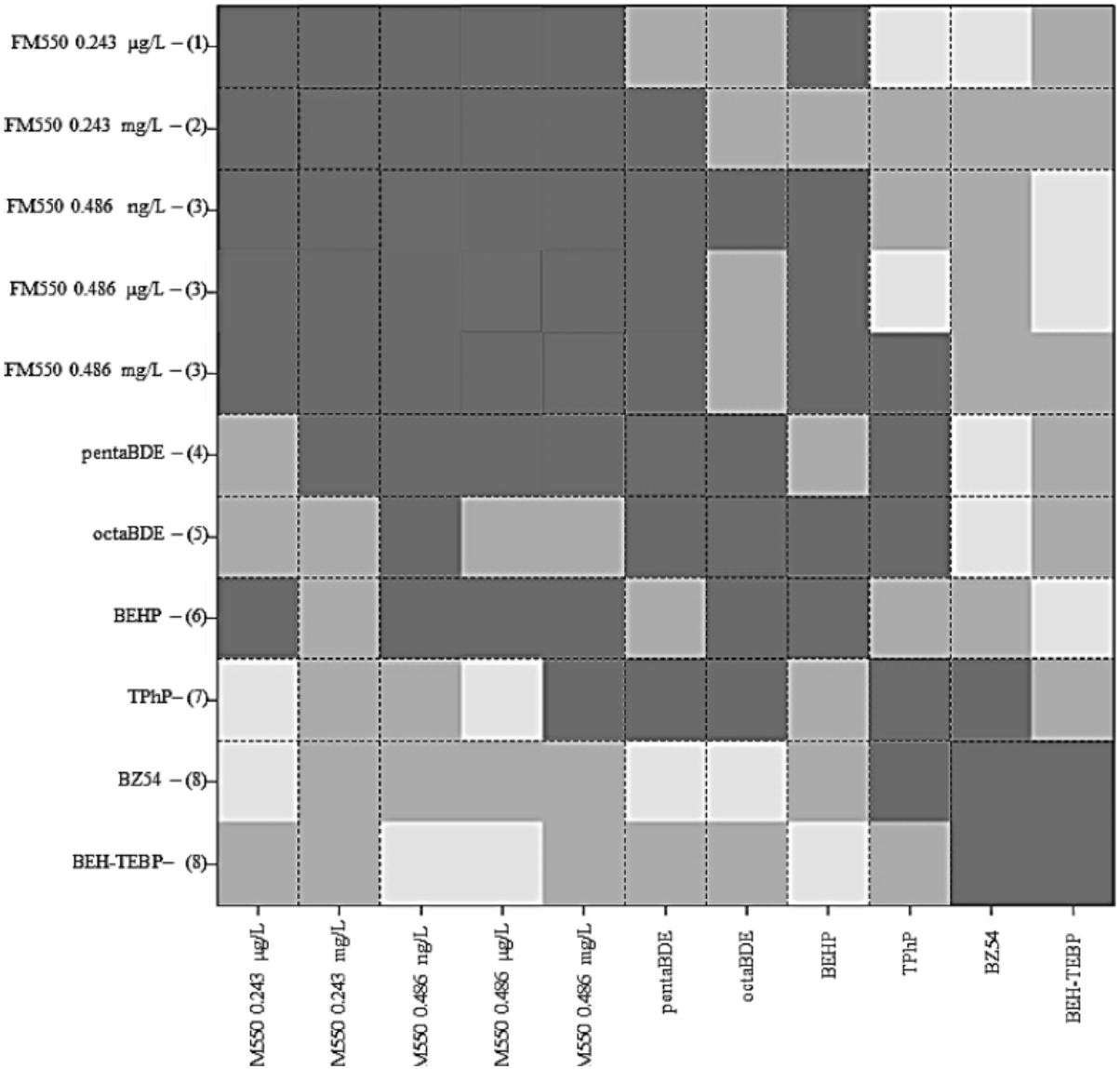
Questions

- Do the liophilic compounds cause similar effects? (narcosis)
- Can we use “omics” to elucidate mechanisms?

Methods

- Acute toxicity tests (48 hour, DMSO solvent)
- cDNA microarray at 1/10 LC_{50}
- HOPACH, KEGG, B2G enrichment analysis and pathway analysis
- Lipidomics (hemolymph) with pentaBDE and FM550
- Accumulation of pentaBDE and FM550
- Metabolomics (HNMR) with pentaBDE and FM550

HOPACH


FM550 0.243 µg/L – (1)
FM550 0.243 mg/L – (2)
FM550 0.486 ng/L – (3)
FM550 0.486 µg/L – (3)
FM550 0.486 mg/L – (3)
pentaBDE – (4)
octaBDE – (5)
BEHP – (6)
TPhP– (7)
BZ54 – (8)
BEH-TEBP– (8)
M550 0.243 µg/L
M550 0.243 mg/L
M550 0.486 ng/L
M550 0.486 µg/L
M550 0.486 mg/L
pentaBDE
octaBDE
BEHP
TPhP
BZ54
BEH-TEBP

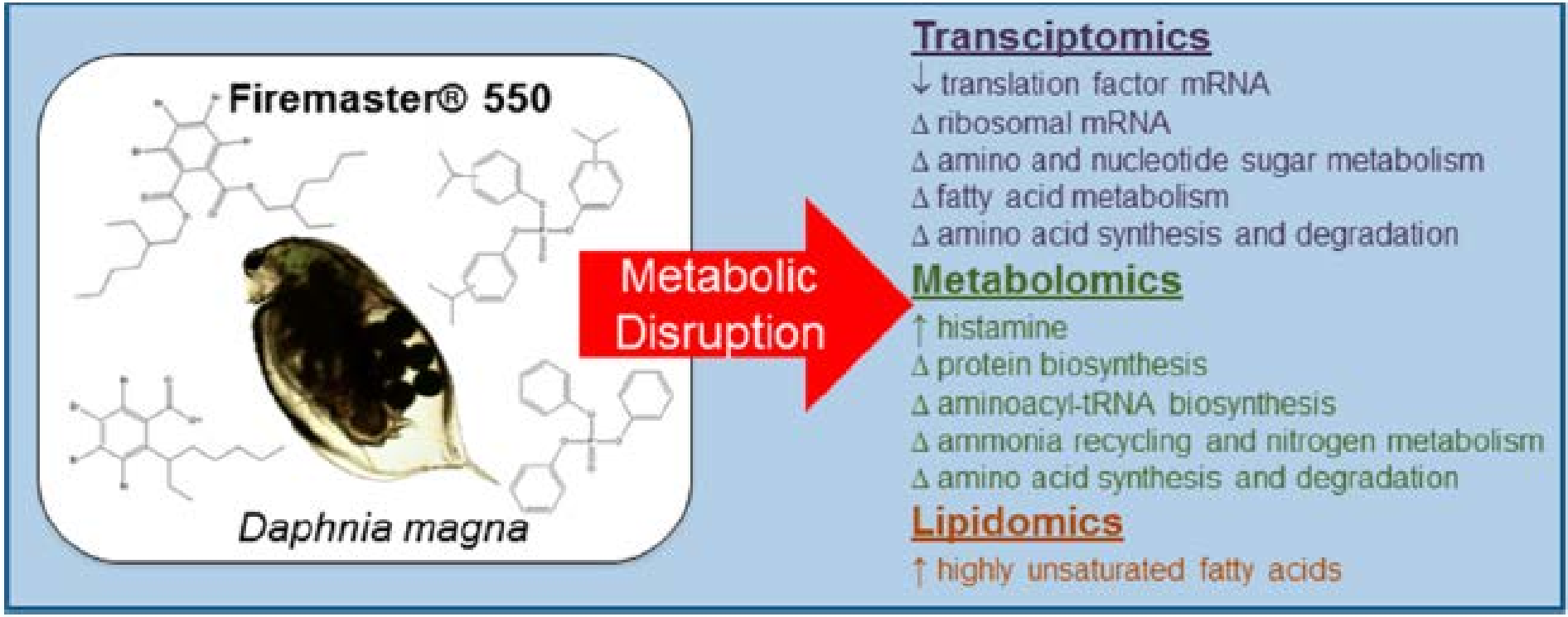
KEGG Pathway Analysis

Table 1. KEGG Pathway Analyses of Gene Transcription Data Show That Exposure to Flame-Retardants Largely Affected Different Biological Pathways[a]

	biological pathways affected by exposure to different chemical flame-retardants					
KEGG biological pathway	FM550	octaBDE	pentaBDE	BZ54	BEH-TEBP	BEHP
ribosome	0.061		0.030			
glycosphingolipid biosynthesis		0.086			0.089	
spliceosome			0.066			
pyrimidine metabolism			0.002			
proteasome			0.074			
WNT signaling pathway				0.089		
porphyrin and chlorophyll metabolism				0.020	0.026	
glycosaminoglycan degradation				0.052		
hedgehog signaling pathway				0.052		
arginine and proline metabolism					0.083	
tyrosine metabolism					0.048	0.042
phenylalanine metabolism					0.044	

[a]Numbers in this graph represent the *p*-value of each analysis. Because annotation of the array is limited, *p*-values up to 0.1 were included.

Firemaster 550



Patisaul et al. *J Biochem Mol Toxicol*. 2013 Feb; 27(2): 124–136: FM550 is obesogen in rats

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Christine Tran
Nadeek Karuaratne
Don Pham
Xin Xin Lin

Flame-retardants

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Photo credit: Lawrence Berkeley Nat'l Lab - Roy Kaltschmidt, photographer