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Update on NTP Studies of Glyphosate

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NTP Glyphosate and Glyphosate Formulations Research

National Toxicology Program
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NTP Glyphosate and Glyphosate Formulations Research

http://ntp.niehs.nih.gov/go/805231

Glyphosate is the most widely used herbicide in the United States and worldwide. It is applied as a formulation (or mixture) with other substances that help the plant absorb the glyphosate. Glyphosate acts as an herbicide by preventing susceptible plants from making proteins that are needed for growth. Over the past 25 years, use of glyphosate has risen dramatically due to development of glyphosate-resistant genetically modified crops. Most people are exposed to glyphosate by ingestion of food or water containing glyphosate residues. Individuals who regularly handle glyphosate products as part of their occupation may experience higher exposures.

There is considerable public interest in the potential health risks to humans from exposure to glyphosate. In 1992, NTP reported that rodents exposed to glyphosate in feed showed little evidence of toxicity, and there was no evidence of glyphosate causing genotoxicity, or damage to DNA.

Recently, several public health agencies have evaluated the scientific literature to identify whether exposure to glyphosate is a cancer hazard for humans.

- In March 2015, the International Agency for Research on Cancer (IARC) concluded that glyphosate is a probable human carcinogen based on evidence from studies in humans and experimental animals. The IARC evaluation also reported that glyphosate-based formulations are generally more toxic than glyphosate alone.
- In November 2015, the European Food Safety Authority concluded that glyphosate is unlikely to pose a carcinogenic hazard to humans.
- In May 2016, the Joint Food and Agricultural Organization of the United Nations/World Health Organization Meeting on Pesticide Residues concluded that glyphosate is unlikely to pose a carcinogenic risk to humans from exposure in the diet.
- Currently, the United States Environmental Protection Agency (EPA) is completing a new human health risk assessment on glyphosate including an evaluation of its carcinogenic potential.

Due to the different interpretations of the scientific evidence regarding potential health risk for humans, public concern for glyphosate use and exposure, and reported differences in toxicity of glyphosate products, NTP is undertaking additional research to investigate the potential genetic and mechanistic toxicity of glyphosate and glyphosate formulations. NTP will also examine the published scientific literature for information about effects of glyphosate on non-cancer outcomes.

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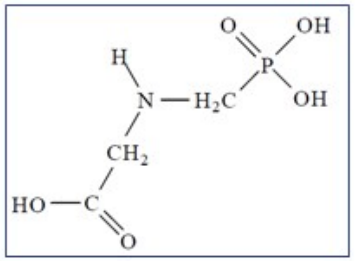
Glyphosate and Glyphosate based formulations

- Broad-spectrum herbicide
- Inhibits an amino acid biosynthetic pathway that is specific to plants.
- Use has risen dramatically in past 25 years due to the use of glyphosate resistant GMO's.
- IARC has listed glyphosate as a probably carcinogenic to humans (2a).
  - *limited evidence of carcinogenicity in humans for non-Hodgkin lymphoma*
  - *sufficient evidence of carcinogenicity in experimental animals*
  - Strong evidence that glyphosate causes genotoxicity and carcinogenicity
- EPA, and EFSA believe glyphosate is unlikely to be carcinogenic to humans

Challenges

Glyphosate



CASRN 1071-83-6

- Glyphosate vs Formulations
  - Rodent cancer studies of pure glyphosate vs epidemiology studies of formulations
- Mechanistic data
  - Unclear how a modified amino acid (glycine + phosphate) would induce oxidative stress leading to genotoxicity.
    - No structural alerts for genotoxic activity using *in silico* prediction programs
  - Formulations are made up of detergent-like ingredients
    - Is oxidative stress induced by these products causing cell death or is the oxidative stress due to cell death caused by these products?

NTP Studies on Glyphosate

- Toxicity Studies of Glyphosate (CASRN 1071-83-6) Administered in Dosed Feed to F344/N Rats and B6C3F1 Mice (1992).
  - Not mutagenic in *Salmonella* (+/- rat liver S9)
  - 13-Week Study
    - High dose of 5% in the diet (≈ 3-12 g/kg/d)
  - Rats
    - At high dose: Decrease body weight, sperm counts, and increased estrous cycle
    - Parotid and submandibular cytoplasmic alterations (Dose dependent)
  - Mice
    - Did not induce micronuclei
    - Parotid and submandibular cytoplasmic alterations (Dose dependent)

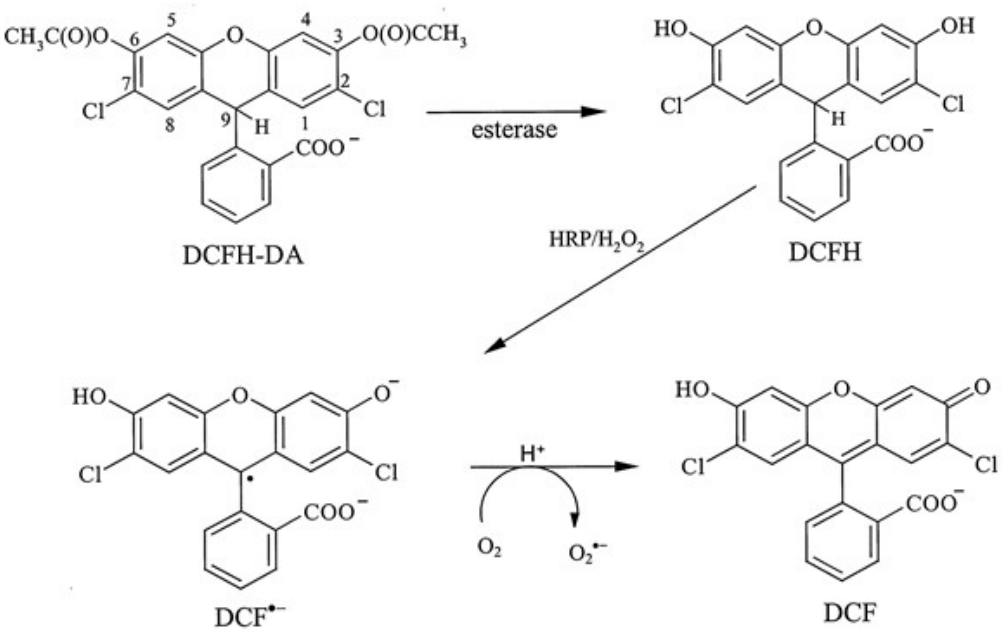
Specific Aims

- Compare the effects of glyphosate to the effects of glyphosate formulations using measures of genotoxicity, oxidative stress, and cell viability.
- Compare the dose response relationships between oxidative stress, genotoxicity, and cell viability.
- Are there other adverse effects of glyphosate and its formulations that require further evaluation?

Cell Based Systems

- HaCaT
  - Immortalized human keratinocytes available from ThermoScientific. Two of the *in vitro* studies on glyphosate and oxidative stress cited by IARC used this cell line.
- HepaRG
  - Derived from human hepatocellular carcinomas and can be cultured to have relatively active xenobiotic metabolism capability.
- TK6
  - Human lymphoblastoid cells that will be used in the genotoxicity companion studies conducted through the Genetic Toxicity Testing contract.

2′,7′-dichlorodihydrofluorescein diacetate



Scheme 2. The proposed mechanism for the HRP-catalyzed DCFH oxidation to DCF

DCFH-DA used in many in vitro studies to look at ROS.

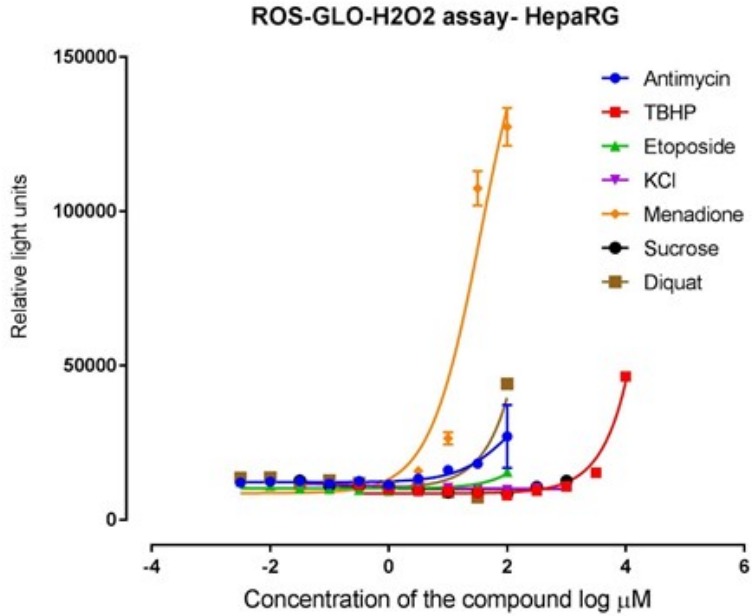
NADH and GSH, which are present in most biological samples, amplifies the DCFH2 oxidation by excited DCF exposed to light

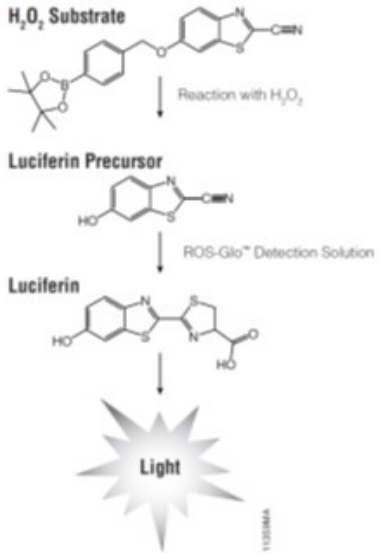
The only way that DCFH2 can be oxidized by H_2O_2 occurs when H_2O_2 reacts with peroxidases or trace metals.

An increase in DCF flourescence does not always indicate an increase in ROS.

Oxidative Stress Assays

Biological Endpoint	Assay	Supplier	Notes
Oxidative Stress	Amplex Red	ThermoScientific	Detects hydrogen peroxide, a reactive oxygen species that causes oxidative damage
Oxidative Stress	ROS-Glo	Promega	Detects (in spent cell culture medium) hydrogen peroxide, a reactive oxygen species that causes oxidative damage
Oxidative Stress	Dihydroethidium	ThermoScientific	Detects superoxide, a reactive oxygen species that causes oxidative damage
Oxidative Stress	Immuno-spin trapping of DMPO adducts	R. Mason Laboratory at NIEHS (Mason, 2016)	Detects evidence of protein damaged by reactive oxidant species
DNA Damage	Homogenous Time-Resolved Fluorescence (HTRF) P-H2AX S139 Sandwich Immunoassay	Cisbio	Detect whether test articles cause DNA damage
Cell Viability	CellTiter-Glo	Promega	Determine concentrations at which test articles cause reduction in cell viability
MMP	JC-10	ThermoScientific	Evaluates Mitochondrial membrane potential

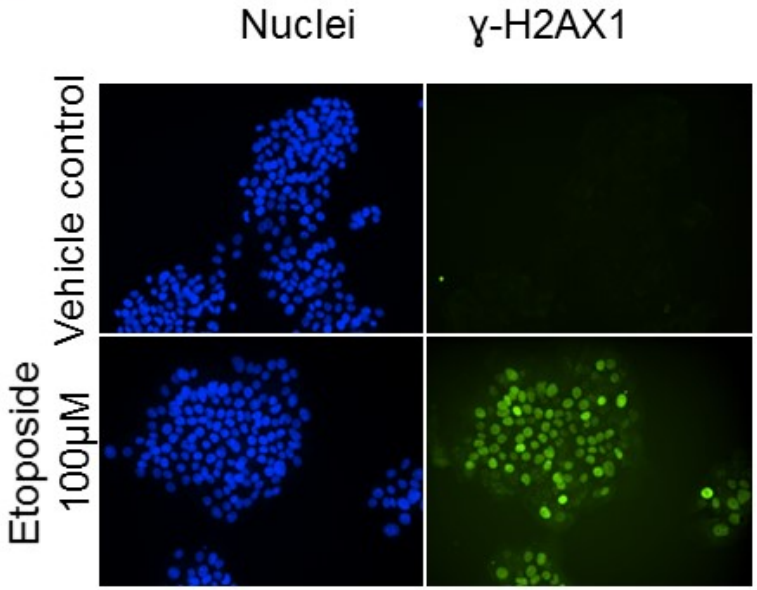




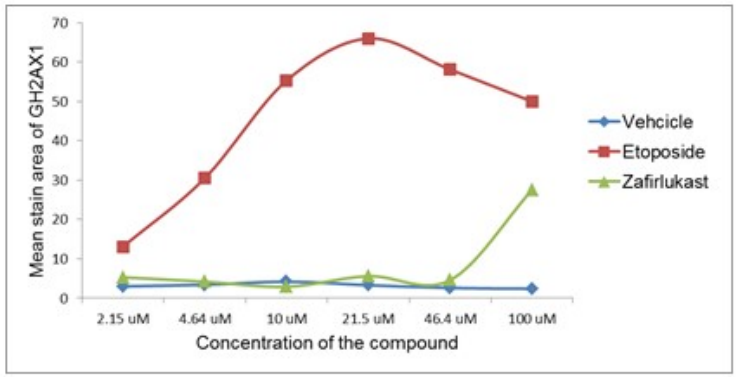
ROS-GLO (Promega) assay chemistry

- The variety of ROS generated in cell cultures or enzyme reactions includes superoxide, hydroxyl radical, singlet oxygen and H2O2.
- H2O2 is convenient to assay because it has the longest half-life of all ROS in cultured cells. In addition, various ROS are converted to H2O2 within cells. For example, superoxide dismutase converts superoxide to O2 and H2O2.
- A change in H2O2 can reflect a general change in the ROS level.

ɣ-H2AX1 Assay



HepaRG



24h dosing

- Early cellular response to Double-strand-breaks, thought to be due to superoxide anion radicals
- Discrete nuclear foci are formed as a result of H2AX phosphorylation

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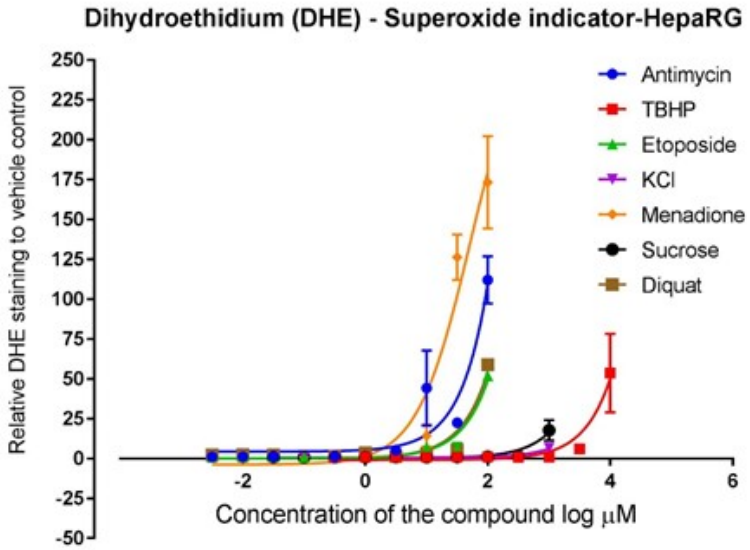
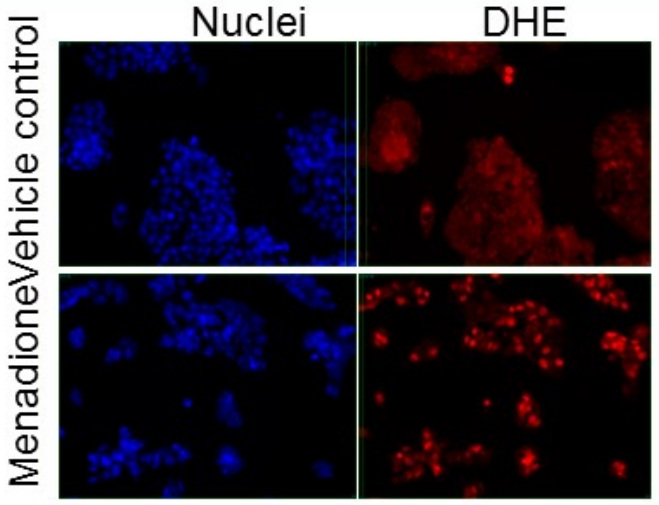
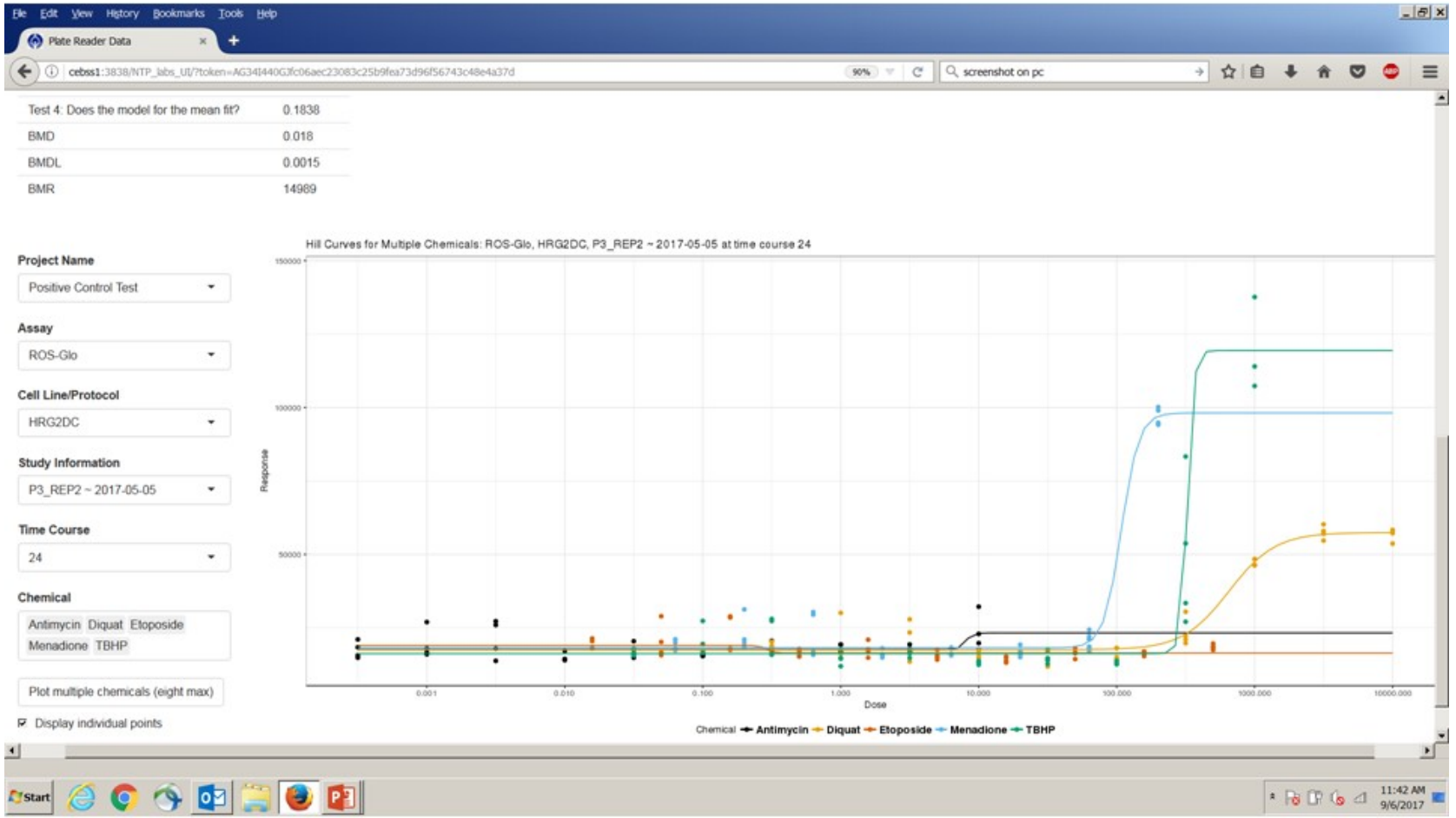




- Dihydroethidium (DHE) can freely permeate cell membranes
- DHE upon reaction with superoxide anions forms a red fluorescent product (ethidium) which intercalates with DNA.

Data Analysis Pipeline in CEBS





What has been done to date?

- Positive and negative controls have been evaluated in HaCaT, HepaRG and HaCaT cells.
- Formulations and actives have been run in HaCaT and HepaRG cells three times and data being analyzed.
- We are in final stages of the data analysis pipeline and visualization tool. The visualization tool will be available on line when the report is released.
- When studies are complete we will publish as an NTP Research Report. (Anticipated sometime in late spring/ early summer).

Genetic Toxicity Testing

Informed by screening efforts at NTPL

- In vitro assays (glyphosate, AMPA, and at least one formulation)
  - Bacterial mutagenicity assays using 5 strains
  - In vitro micronucleus assay with human lymphoblastoid TK6 cells
  - In vitro comet assay with TK6 cells
    - Comet assay can also be performed as a modified comet assay to detect DNA damage from oxidative stress.
- In vivo assays (glyphosate and a formulation)
  - Rats and mice via gavage
  - Combined micronucleus and comet assay
    - Comet assay can also be performed as a modified comet assay to detect DNA damage from oxidative stress.

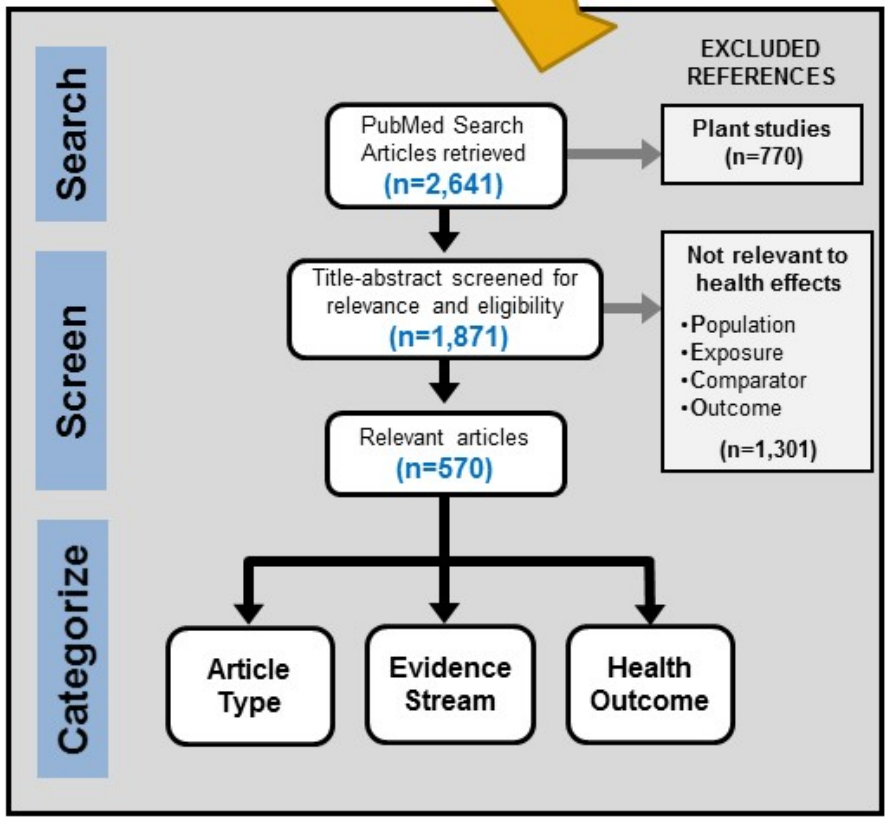
Are there other endpoints of concern?

- NTP is conducting a screening-level analysis of the existing literature using text mining and machine-learning approaches.
- Provide an overview of available literature for all human health outcomes related to glyphosate exposure.

Glyphosate Literature

OHAT Actively Monitoring for Health Effects Studies

- Literature Search and Screening
  - Monitor extent of evidence
  - Multiple databases
    - PubMed in figure
  - Refining search approach
    - Manual screening + machine-learning



Glyphosate Literature

OHAT Actively Monitoring for Health Effects Studies

- Evidence Mapping
  - Categorize by
    - Major Health Effects
    - Evidence Stream
      - Human
      - Animal
      - In vitro exposure
      - In silico
  - Interactive Tableau graphic
    - Working to optimize
      - Workflow
      - Utility of content

Health Effect Categories	Review or Commentary	Animal	Human	In silico	In Vitro
Acute Toxicity	4	39	61		7
ADME/Exposure	3	11	16		11
Cancer	17	49	13		29
Cardiovascular	3	9	21		5
Clinical Chemistry	1	52	17	1	19
Endocrine	1	34			13
Gastrointestinal	3	10	17		6
Growth and Development	7	79	10		1
Hematological and Immune		53	13		16
Hepatic	1	68	6		13
Mortality	1	94	19		
Musculoskeletal		26	3		4
Neurological and Sensory	3	70	24		19
Nutrition and Metabolic		31	1		4
Renal	1	16	21		3
Reproductive	5	53	10		12
Respiratory	2	22	32		3

Summary

- NTP is performing in vitro experiments examining the role of oxidative stress and genotoxicity in the toxicity of glyphosate and glyphosate formulations.
- In vivo studies may be performed based on in vitro results.
- NTP is conducting a screening-level analysis of the existing literature using text mining and machine-learning approaches.

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