U.S. EPA Meeting on Cr(VI) Research

August 10, 2016

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Outline

1. **Background and design of MOA research project**

2. **Summary of MOA research findings for Cr(VI)**

3. **Open discussion on research findings**
   a) Q & A
   b) New MOA data
   c) New PK data
   d) Other topics

Summary

- Tumors observed in the NTP study occurred only at very high doses
- Pharmacokinetic data indicate non-linearities in Cr(VI) disposition
- Precedent for non-genotoxic/threshold MOA for SI tumors
- Cr(VI) does not induce genotoxicity in target tissues
- Substantial evidence for a cytotoxicity/regenerative hyperplasia MOA

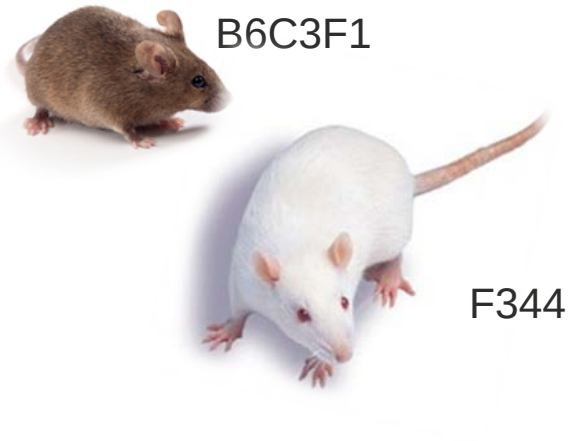
NTP Cr(VI) and Cr(III) Bioassays (2008)

NTP Cr(VI) drinking water study

- 5 to 180 ppm
- Rare tumors appeared late in the study

  Mice: adenomas and carcinomas of SI (≥30 ppm)

  Rats: SCC in oral cavity (180 ppm)



NTP Cr(III) 2 year feeding study

- 2,000 to 50,000 ppm
- No significant effects in either species

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NTP Cr(VI) and Cr(III) Bioassays (2008)

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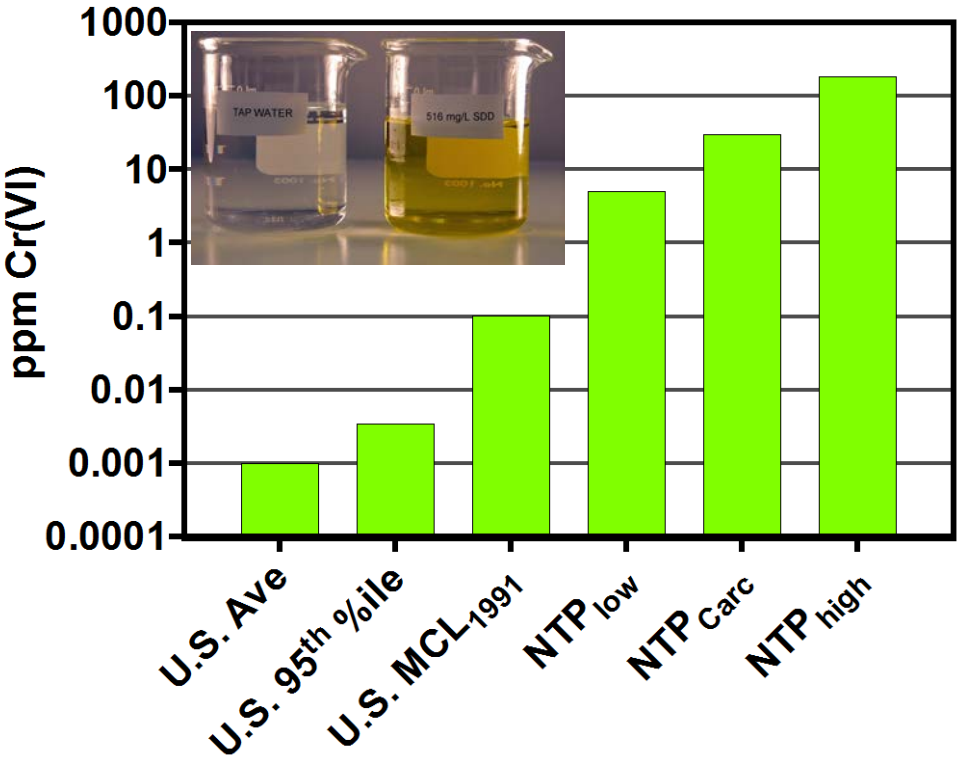

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Cr(VI) MOA Research Project

Replicated aspects of NTP Cr(VI) study

- Same strains (B6C3F1 mice, F344 rats)
- Same doses, plus two lower doses (including MCL)
- Data collected after 7 and 90 days of exposure

Specifically investigated target tissue of small intestine and oral mucosa

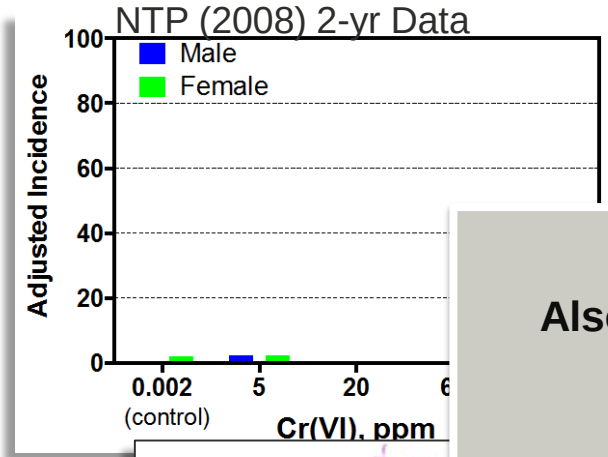
- Biochemistry
- *In vivo* genotoxicity
- Histopathology
- Toxicogenomics
- *In vitro* genotoxicity

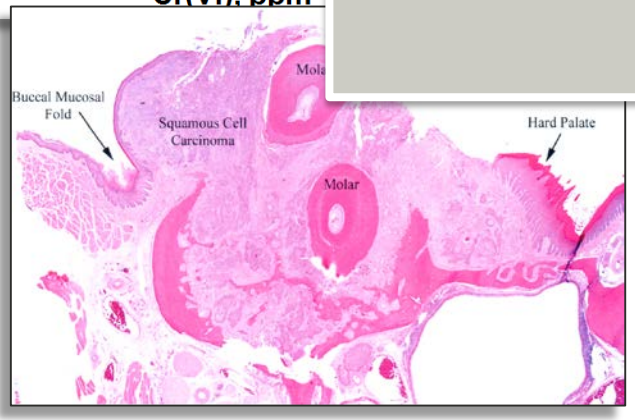
Evaluated toxicokinetics

- Measured rates and capacity of Cr(VI) reduction to Cr(III) in human and rodent stomach contents
- Developed Physiologically-based Pharmacokinetic (PBPK) Models

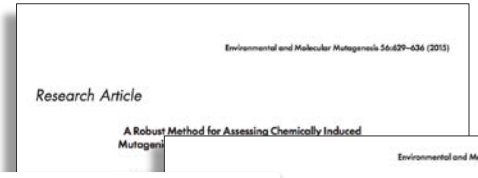
Results used to inform derivation of toxicity values

MOA Research on Oral Tumors in Rats (Overview)





Also have new toxicogenomic data we can discuss later today….

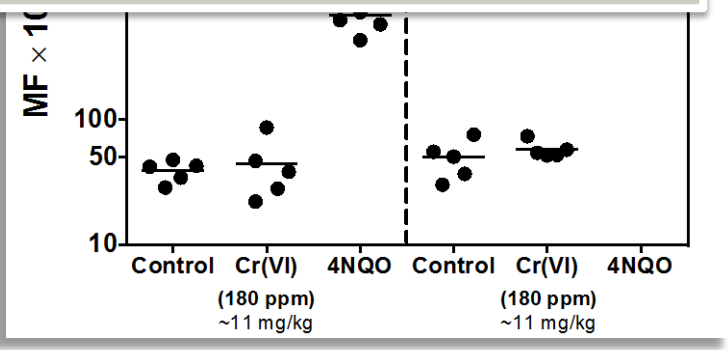

Environmental and Molecular Mutagenesis 56:629–636 (2015)

Research Article

A Robust Method for Assessing Chemically Induced


Environmental and Molecular Mutagenesis 56:621–628 (2015)

Assessment of the Mutagenic Potential of Cr(VI) in the Oral Mucosa of Big Blue® Transgenic F344 Rats



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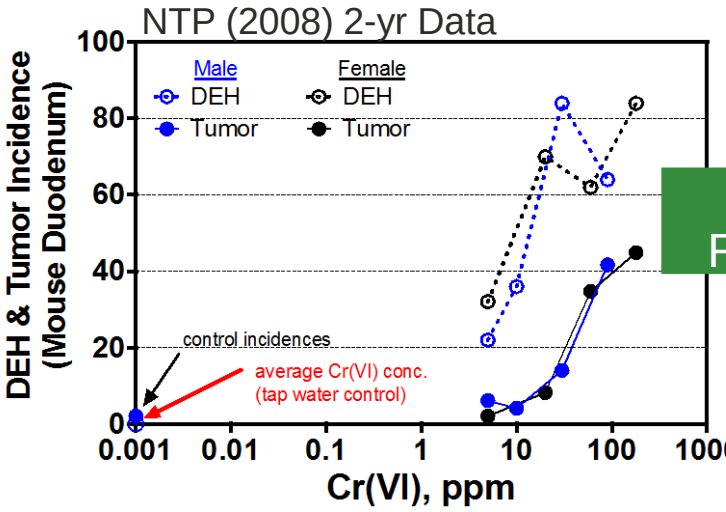
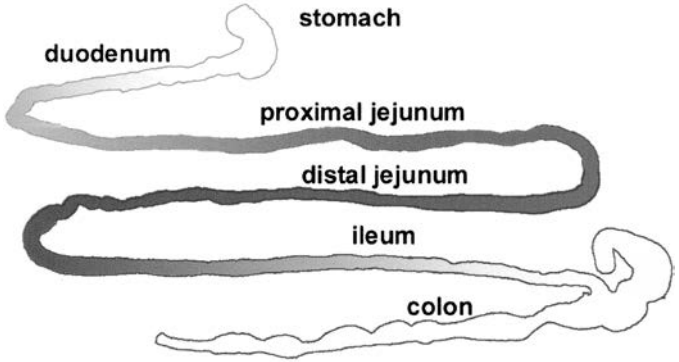
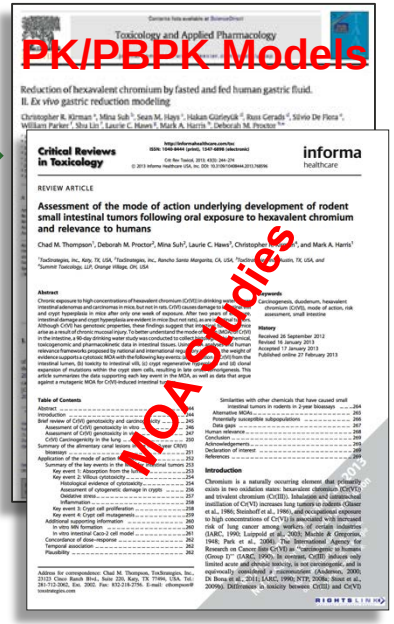
MOA Research on Duodenal Tumors in Mice (Overview)





MOA Research



RfD for Cancer

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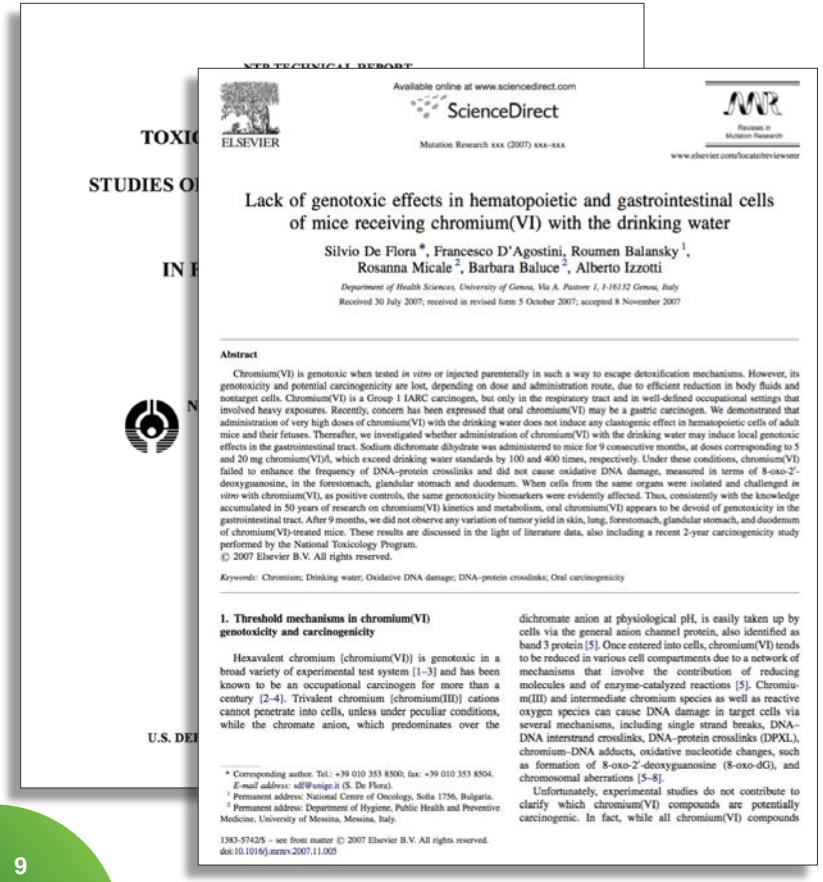
Early Suggestions of Nonlinear Mechanisms/Pharmacokinetics



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Mutation Research xxx (2007) xxx–xxx
www.elsevier.com/locate/reviewsmr

Lack of genotoxic effects in hematopoietic and gastrointestinal cells of mice receiving chromium(VI) with the drinking water

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Received 30 July 2007; received in revised form 5 October 2007; accepted 8 November 2007

Abstract

Chromium(VI) is genotoxic when tested *in vitro* or injected parenterally in such a way to escape detoxification mechanisms. However, its genotoxicity and potential carcinogenicity are lost, depending on dose and administration route, due to efficient reduction in body fluids and nontarget cells. Chromium(VI) is a Group 1 IARC carcinogen, but only in the respiratory tract and in well-defined occupational settings that involved heavy exposures. Recently, concern has been expressed that oral chromium(VI) may be a gastric carcinogen. We demonstrated that administration of very high doses of chromium(VI) with the drinking water does not induce any clastogenic effect in hematopoietic cells of adult mice and their fetuses. Thereafter, we investigated whether administration of chromium(VI) with the drinking water may induce local genotoxic effects in the gastrointestinal tract. Sodium dichromate dihydrate was administered to mice for 9 consecutive months, at doses corresponding to 5 and 20 mg chromium(VI)/l, which exceed drinking water standards by 100 and 400 times, respectively. Under these conditions, chromium(VI) failed to enhance the frequency of DNA–protein crosslinks and did not cause oxidative DNA damage, measured in terms of 8-oxo-2′-deoxyguanosine, in the forestomach, glandular stomach and duodenum. When cells from the same organs were isolated and challenged *in vitro* with chromium(VI), as positive controls, the same genotoxicity biomarkers were evidently affected. Thus, consistently with the knowledge accumulated in 50 years of research on chromium(VI) kinetics and metabolism, oral chromium(VI) appears to be devoid of genotoxicity in the gastrointestinal tract. After 9 months, we did not observe any variation of tumor yield in skin, lung, forestomach, glandular stomach, and duodenum of chromium(VI)-treated mice. These results are discussed in the light of literature data, also including a recent 2-year carcinogenicity study performed by the National Toxicology Program.

Keywords: Chromium; Drinking water; Oxidative DNA damage; DNA–protein crosslinks; Oral carcinogenicity

1. Threshold mechanisms in chromium(VI) genotoxicity and carcinogenicity

Hexavalent chromium [chromium(VI)] is genotoxic in a broad variety of experimental test system [1–3] and has been known to be an occupational carcinogen for more than a century [2–4]. Trivalent chromium [chromium(III)] cations cannot penetrate into cells, unless under peculiar conditions, while the chromate anion, which predominates over the dichromate anion at physiological pH, is easily taken up by cells via the general anion channel protein, also identified as band 3 protein [5]. Once entered into cells, chromium(VI) tends to be reduced in various cell compartments due to a network of mechanisms that involve the contribution of reducing molecules and of enzyme-catalyzed reactions [5]. Chromium(III) and intermediate chromium species as well as reactive oxygen species can cause DNA damage in target cells via several mechanisms, including single strand breaks, DNA–DNA interstrand crosslinks, DNA–protein crosslinks (DPXL), chromium–DNA adducts, oxidative nucleotide changes, such as formation of 8-oxo-2′-deoxyguanosine (8-oxo-dG), and chromosomal aberrations [5–8].

Unfortunately, experimental studies do not contribute to clarify which chromium(VI) compounds are potentially carcinogenic. In fact, while all chromium(VI) compounds

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doi:10.1016/j.mrrev.2007.11.005

- NTP (2008) study authors only observed diffuse epithelial hyperplasia (DEH) in mice
  - Characterized DEH as secondary to mucosal injury in both 13-wk and 2-yr studies
- Silvio De Flora (2008) noted:
  - lack of tumors or genotoxic lesions in intestines of mice exposed to ≤20 ppm Cr(VI) for 9 mo
  - "…the increase of intestinal tumors in the NTP study was only observed in mice and not in rats, and only at very high doses, unrealistic for human exposures. This clearly implies occurrence of threshold mechanisms…"

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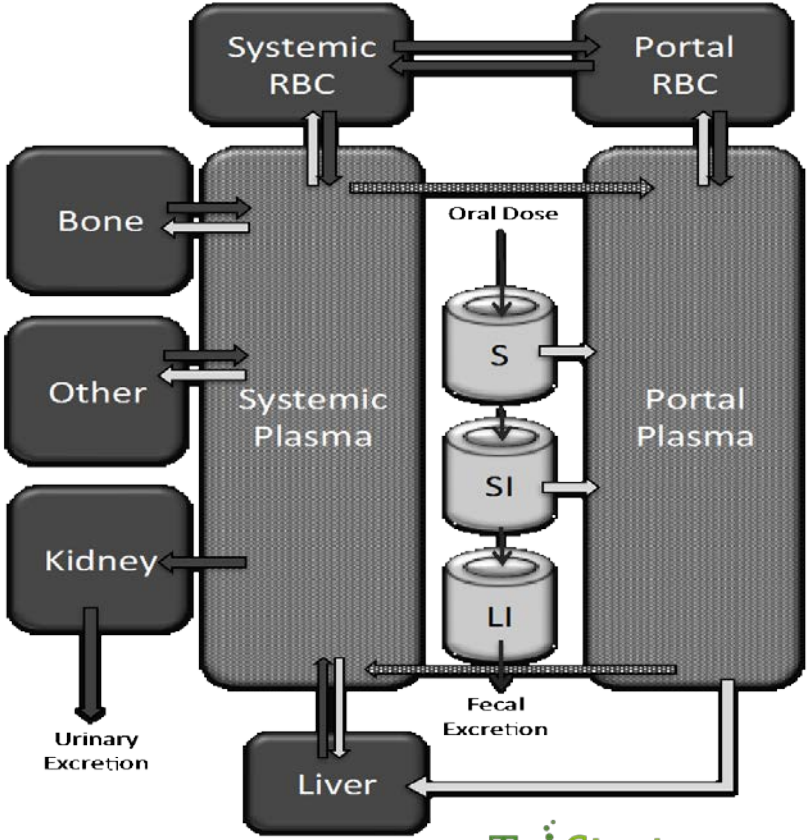
PBPK Model Developed to Address Nonlinearities

- Provide target tissue dosimetry (to mouse small intestines) instead of administered dose
- Simulate rodents exposed to CrVI under conditions of the NTP cancer bioassay (NTP, 2008)
- Support risk assessment decisions regarding human populations exposed to CrVI
  - Improve interspecies extrapolation
  - Improve high-to-low dose extrapolation
  - Assessment of sensitive subpopulations due to PK factors



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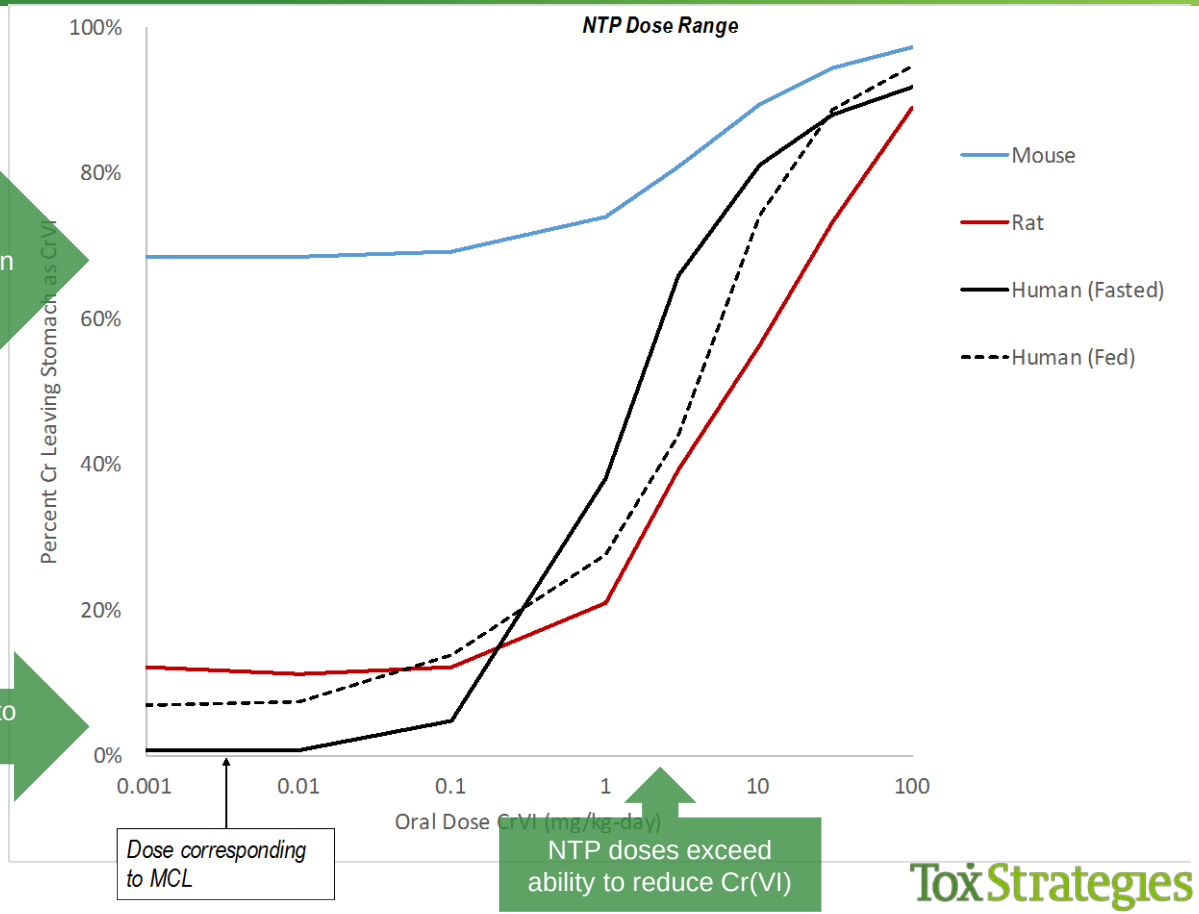
Nonlinearities and Species Differences in Dosimetry to SI

High gastric motility and high pH in mice allows Cr(VI) to escape reduction and move into SI

Most Cr(VI) is reduced prior to passage to SI at low levels



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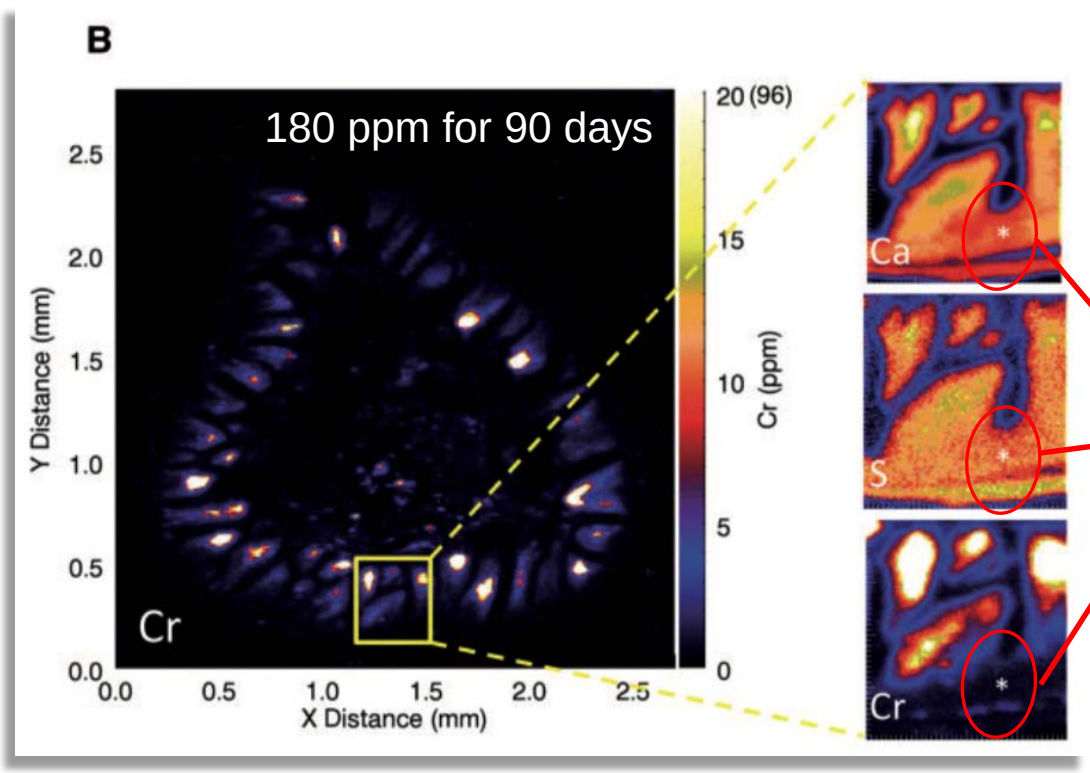
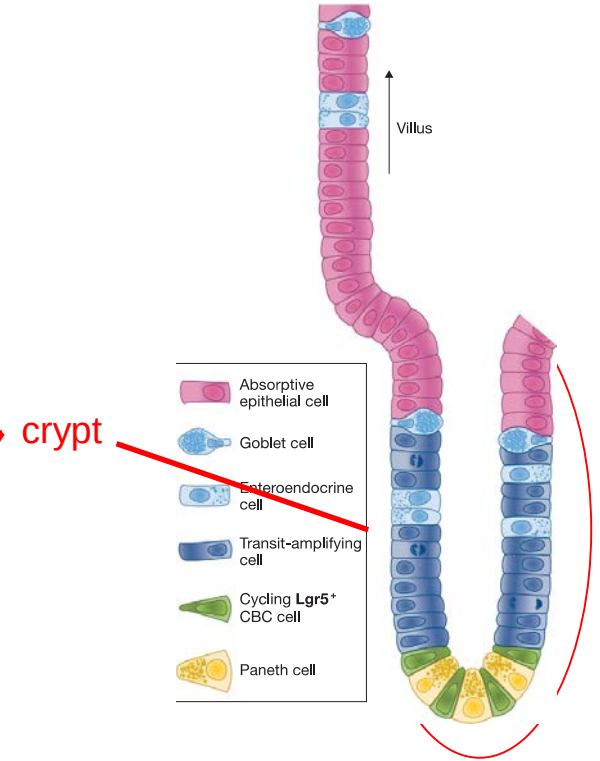
Cr(VI) Entering SI Localizes to Intestinal Villi (Not Crypts)





Thompson et al. (2015) Tox Sci

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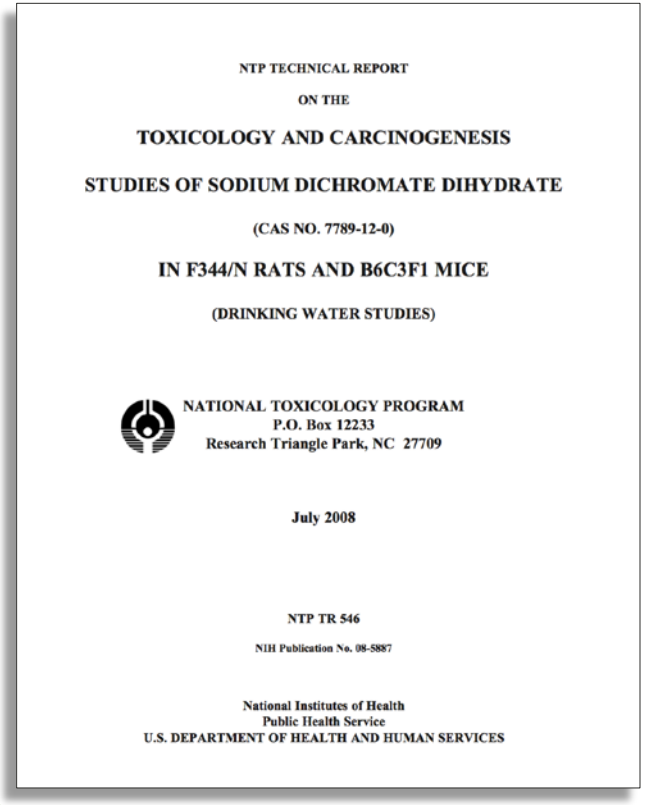
Precedent for Non-mutagenic MOA for SI Tumors



NTP TECHNICAL REPORT

ON THE

TOXICOLOGY AND CARCINOGENESIS

STUDIES OF SODIUM DICHROMATE DIHYDRATE

(CAS NO. 7789-12-0)

IN F344/N RATS AND B6C3F1 MICE

(DRINKING WATER STUDIES)

NATIONAL TOXICOLOGY PROGRAM
P.O. Box 12233
Research Triangle Park, NC 27709

July 2008

NTP TR 546

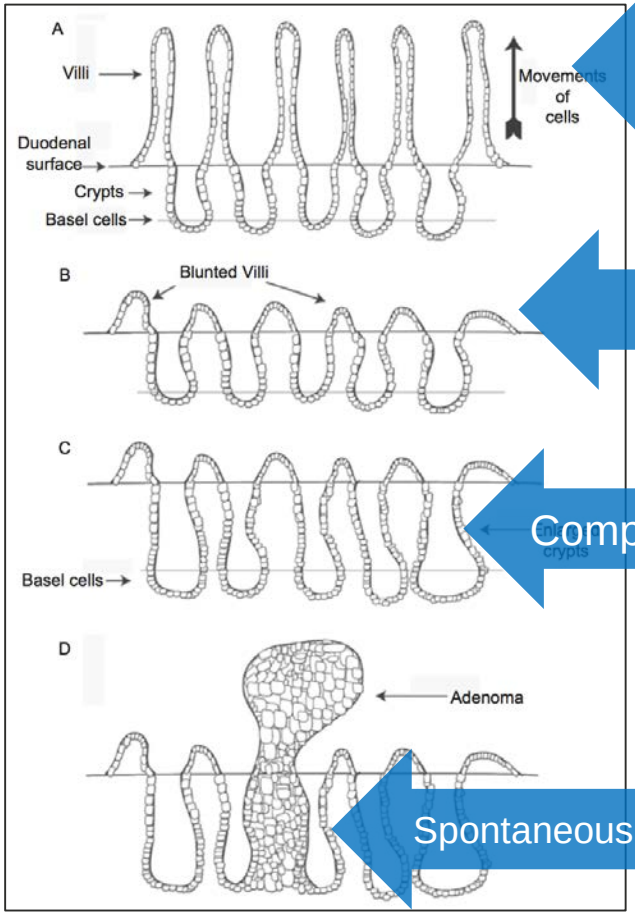
NIH Publication No. 08-5887

National Institutes of Health
Public Health Service
U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES

- NTP study authors noted that **captan** was "the only other study performed by the NTP in B6C3F1 mice in which both benign and malignant intestinal neoplasms of epithelial origin have been definitely attributed to chemical exposure"

- U.S. EPA (2004):
  - "**captan** induces adenomas and adenocarcinomas in the duodenum of the mouse by a nongenotoxic MOA involving cytotoxicity and regenerative cell hyperplasia that exhibits a clear dose threshold...
  - EPA classified captan as "not likely to be a human carcinogen at dose levels that do not cause cytotoxicity and regenerative cell hyperplasia"

Proposed MOA For Captan/Folpet



Source: Cohen et al. (2010) Crit Rev Toxicol 40: 531.

Chemical absorption at villi

Toxicity to villous enterocytes

Compensatory crypt hyperplasia

actually occur concomitantly

Spontaneous mutation leads to tumorigenesis

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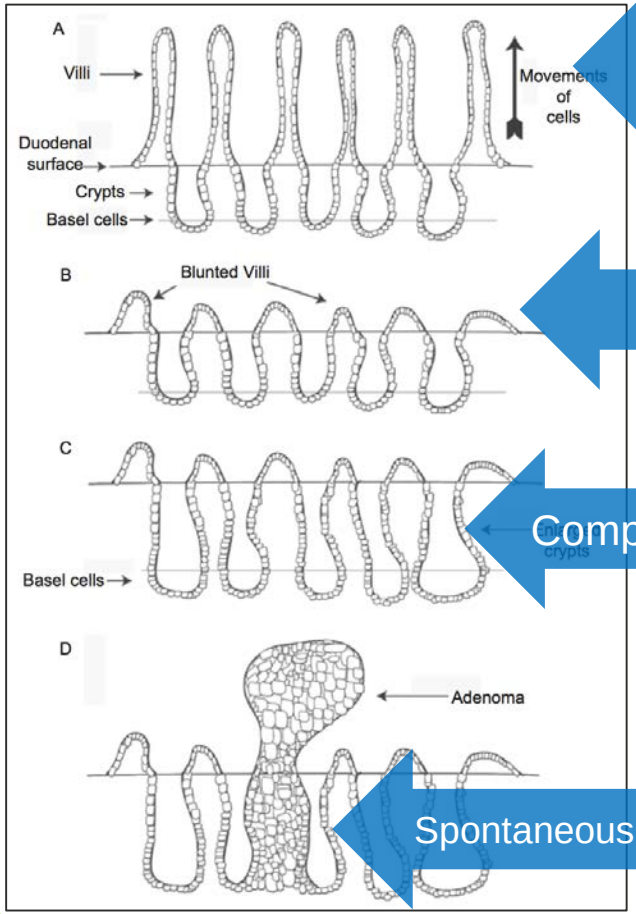
Similarities Between Captan/Folpet and Cr(VI)



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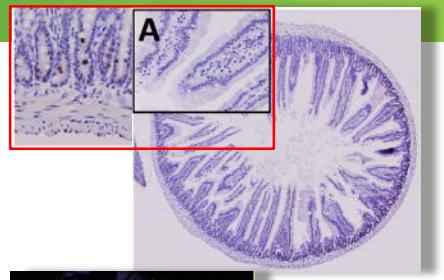
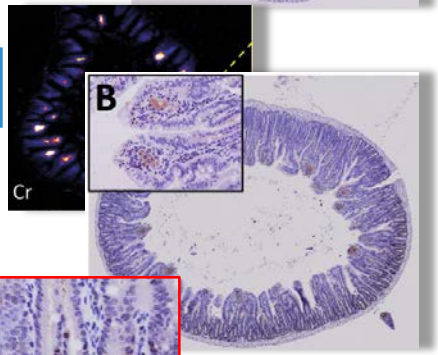
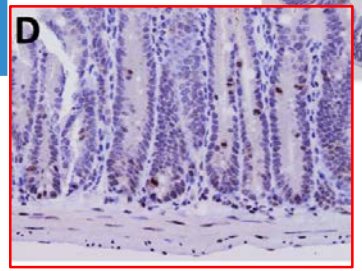


Toxicity to villous enterocytes



Compensatory crypt hyperplasia



Spontaneous mutation leads to tumorigenesis

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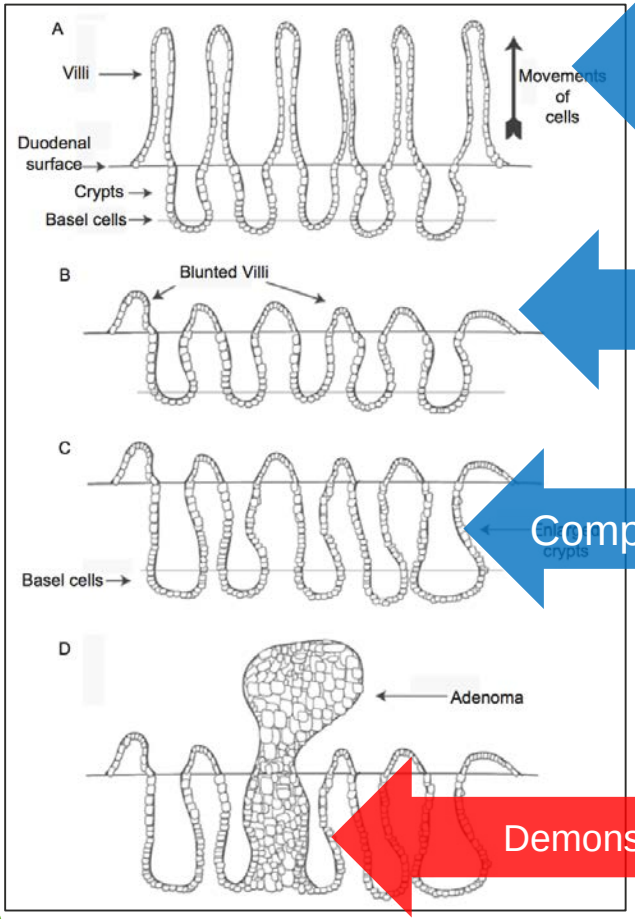
Similarities Between Captan/Folpet and Cr(VI)



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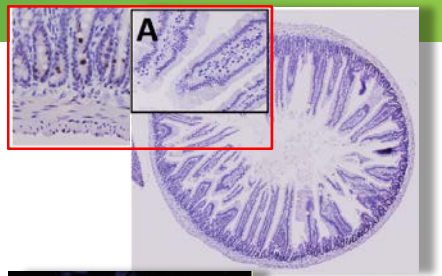
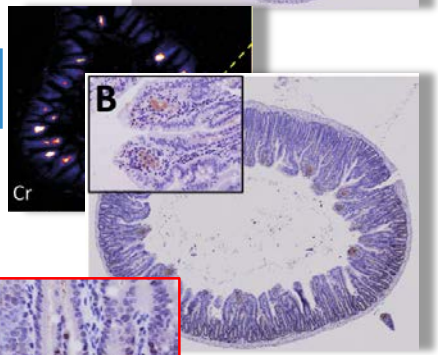
Chemical absorption at villi

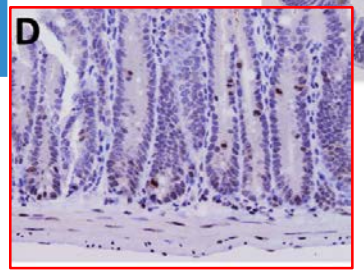
Toxicity to villous enterocytes

Compensatory crypt hyperplasia

Demonstrate lack of genotoxicity…







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IWGT Recommendations for *In Vivo* Genotoxicity Assays

Mutation Research 783 (2015) 66–78

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

IWGT report on quantitative approaches to genotoxicity risk assessment II. Use of point-of-departure (PoD) metrics in defining acceptable exposure limits and assessing human risk☆

James T. MacGregor[a,*], Roland Frötschl[b], Paul A. White[c], Kenny S. Crump[d], David A. Eastmond[e], Shoji Fukushima[f], Melanie Guérard[g], Makoto Hayashi[h], Lya G. Soeteman-Hernández[i], George E. Johnson[j], Toshio Kasamatsu[k], Dan D. Levy[l], Takeshi Morita[m], Lutz Müller[g], Rita Schoeny[n], Maik J. Schuler[o], Véronique Thybaud[p]

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ABSTRACT

This is the second of two reports from the International Workshops on Genotoxicity Testing (IWGT) Working Group on Quantitative Approaches to Genetic Toxicology Risk Assessment (the QWG). The first report summarized the discussions and recommendations of the QWG related to the need for quantitative dose–response analysis of genetic toxicology data, the existence and appropriate evaluation of threshold responses, and methods to analyze exposure-response relationships and derive points of departure (PoDs) from which acceptable exposure levels could be determined. This report summarizes the QWG discussions and recommendations regarding appropriate approaches to evaluate exposure-related risks of genotoxic damage, including extrapolation below identified PoDs and across test systems and species. Recommendations include the selection of appropriate genetic endpoints and target tissues, uncertainty factors and extrapolation methods to be considered, the importance and use of information on mode of action, toxicokinetics, metabolism, and exposure biomarkers when using quantitative exposure-response data to determine acceptable exposure levels in human populations or to assess the risk associated with known or anticipated exposures. The empirical relationship between genetic damage (mutation and chromosomal aberration) and cancer in animal models was also examined. It was concluded that there is a general correlation between cancer induction and mutagenic and/or clastogenic damage for agents thought to act via a genotoxic mechanism, but that the correlation is limited due to an inadequate number of cases in which mutation and cancer can be compared at a sufficient number of doses in the same target tissues of the same species and strain exposed under directly comparable routes and experimental protocols.

☆ The opinions and recommendations expressed in this publication are those of the authors, and do not necessarily reflect those of the institutions with which they may be affiliated.

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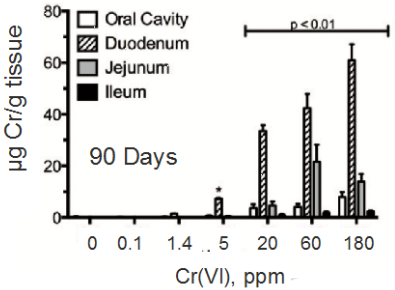
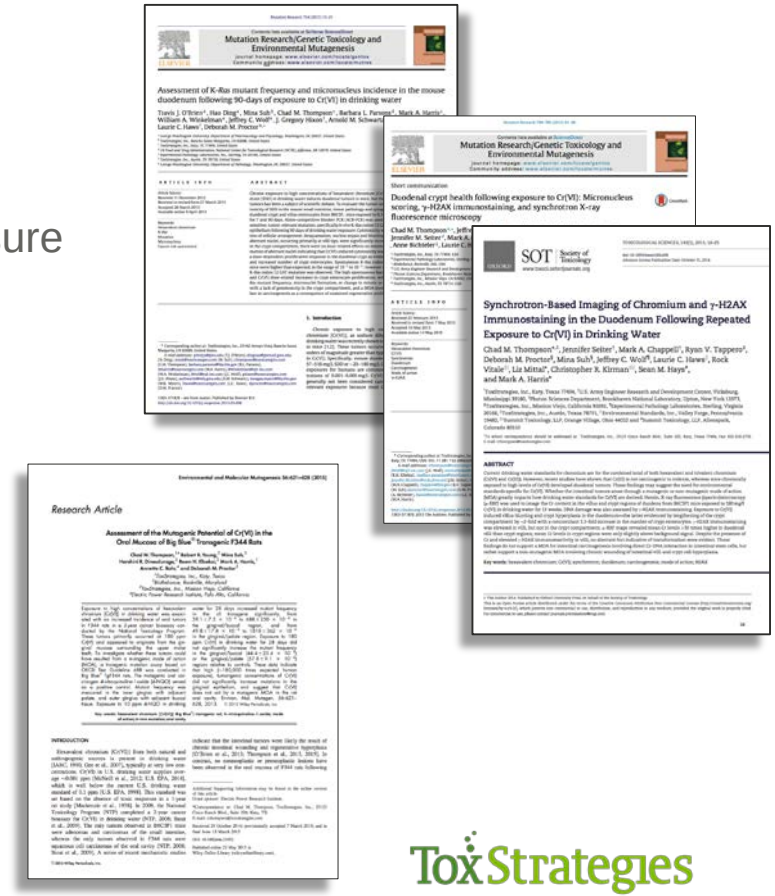
http://dx.doi.org/10.1016/j.mrgentox.2014.10.008
1383-5718/

- Ideally conducted in a proliferative tissue
  - Bone marrow (hematopoietic)
  - Colon
  - Stomach
  - **Small intestine (duodenum)**
- Ideally at site of carcinogenic action
  - **GI tract for Cr(VI)**
- Ideally in tissue with high dosimetry (e.g. site of contact)
  - Stomach
  - Liver
  - **Duodenum for Cr(VI)**

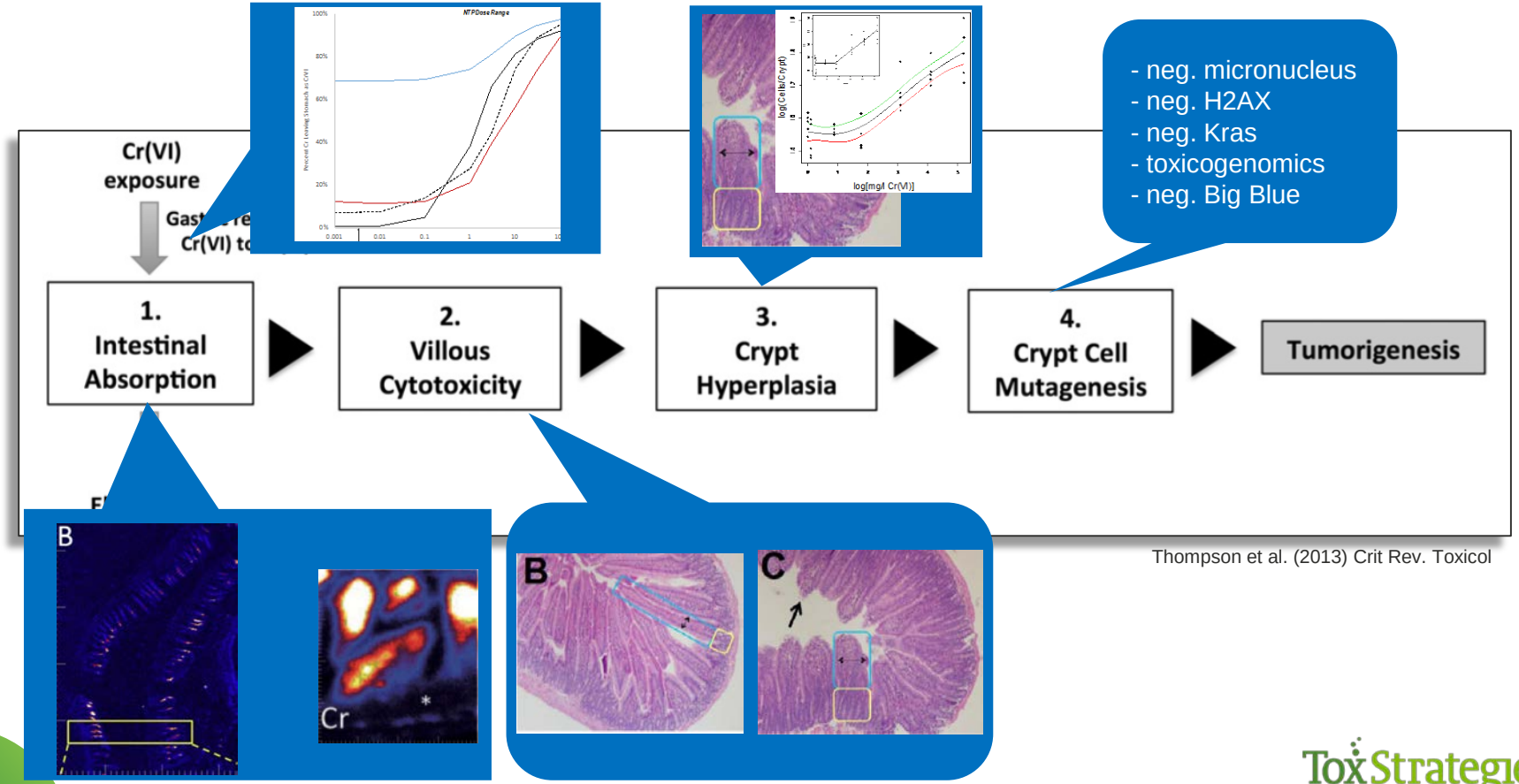


In Vivo Genotoxicity in Target Tissues

- Duodenal MN assays
  - Neg after 7 and 90 days of exposure
- Duodenal γ-H2AX immunostaining
  - No diff from controls at 7 and 90 days of exposure
- *kras* codon 12 GAT MF in duodenum
  - Neg after 90 days of exposure
- XRF microscopy
  - Cr detected in villi (not crypt)
- Oral mucosa mutation assay
  - Neg in Big Blue rats after 28 days of exposure
- Blood MN assays
  - most are neg.



Non-mutagenic MOA for SI Tumors



Thompson et al. (2013) Crit Rev. Toxicol

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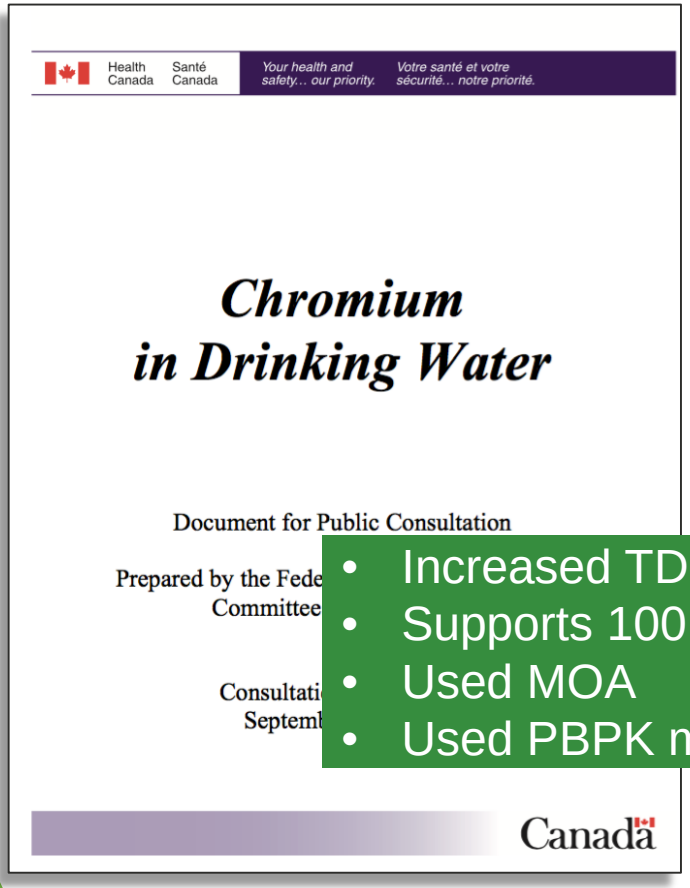
Evidence for Cytotoxic MOA

Modified Bradford-Hill	Supporting Evidence	Potential Inconsistent Data
Dose-response, temporal concordance	• Prolif. @ lower doses than tumors • Prolif. @ 1 wk, 13 wk, 2 yr • Genotoxicity not observed in target tissue (day 8 or 91)	
Consistency, specificity	• Prolif. in multiple mouse studies @ 1 wk, 13 wk • Prolif. in multiple species (mice>>rats) • Mild prolif. ≠ SI tumors • XRF maps: Cr localizes to villi in both species • Crypts line entire intestine, but tumors observed in region of high villous absorption	• Some individual mice with tumors were ”neg” for DEH; however, DEH diagnosis is based on a single single biopsy/slide
Biological plausibility	• Similar MOA for intestinal carcinogens captan and folpet • Crypt stem cells are source of SI cancer	• Villous enterocytes can be dedifferentiated experimentally

Framework adapted from WHO/IPCS Framework on MOA/Species Concordance

Recently Proposed RfDs Protective of Cancer


Health Canada Santé Canada

Your health and safety... our priority. *Votre santé et votre sécurité... notre priorité.*

Chromium in Drinking Water

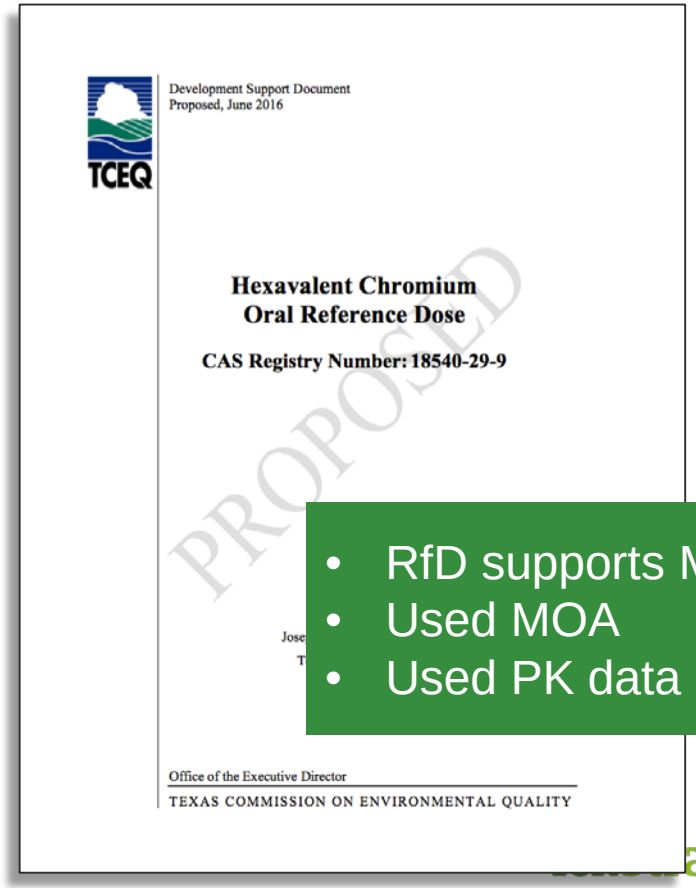
Document for Public Consultation

Prepared by the Fede
Committee

Consultati
Septemb

Canada

- Increased TDI 2-fold
- Supports 100 ppb
- Used MOA
- Used PBPK models


TCEQ

Development Support Document
Proposed, June 2016

Hexavalent Chromium
Oral Reference Dose

CAS Registry Number: 18540-29-9

PROPOSED

Jose
T

Office of the Executive Director

TEXAS COMMISSION ON ENVIRONMENTAL QUALITY

- RfD supports MCL
- Used MOA
- Used PK data

ategies

Summary

- Tumors observed in the NTP study occurred only at very high doses
  - Exceeded ability to reduce Cr(VI) to Cr(III)
- Pharmacokinetic data indicate non-linearities in Cr(VI) disposition
  - Depletion of reducing pools at high doses
- Precedent for non-genotoxic/threshold MOA for SI tumors
  - Captan/folpet determined to act by cytotoxicity and regenerative hyperplasia
- Cr(VI) does not induce genotoxicity in target tissues
  - Neg results in mutation and clastogenicity assays
- Substantial evidence for a cytotoxicity/regenerative hyperplasia MOA
  - NTP study authors indicated hyperplasia 2° to mucosal injury
  - Re-evaluation by pathologists concluded villus toxicity led to crypt hyperplasia
  - MOA data provide consistent support for threshold MOA

Collaborators and Co-authors on MOA Studies

Tox Strategies

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Mark A. Harris
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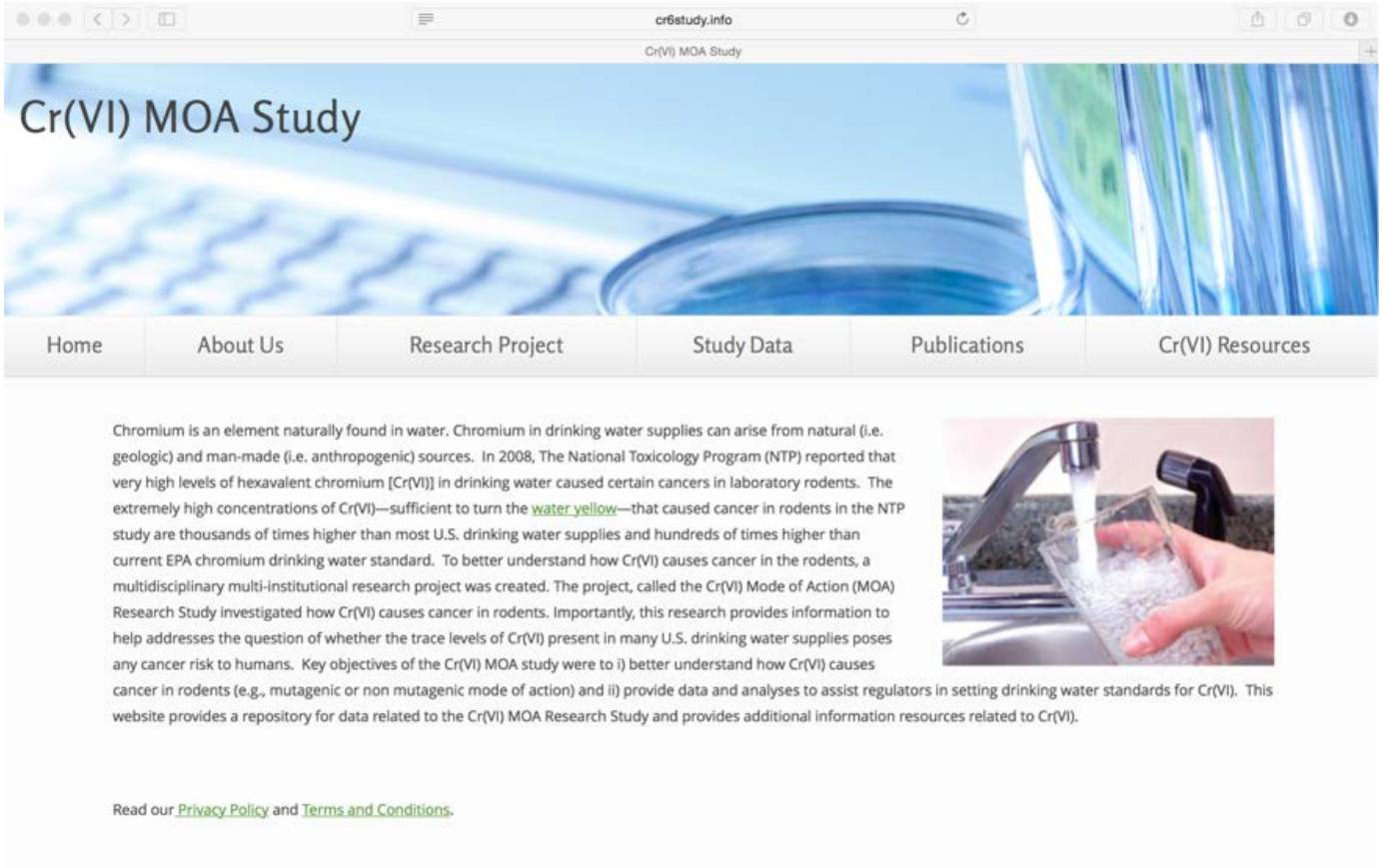
EPL

Jeffrey C. Wolf

Study Transparency: Data Publically Available



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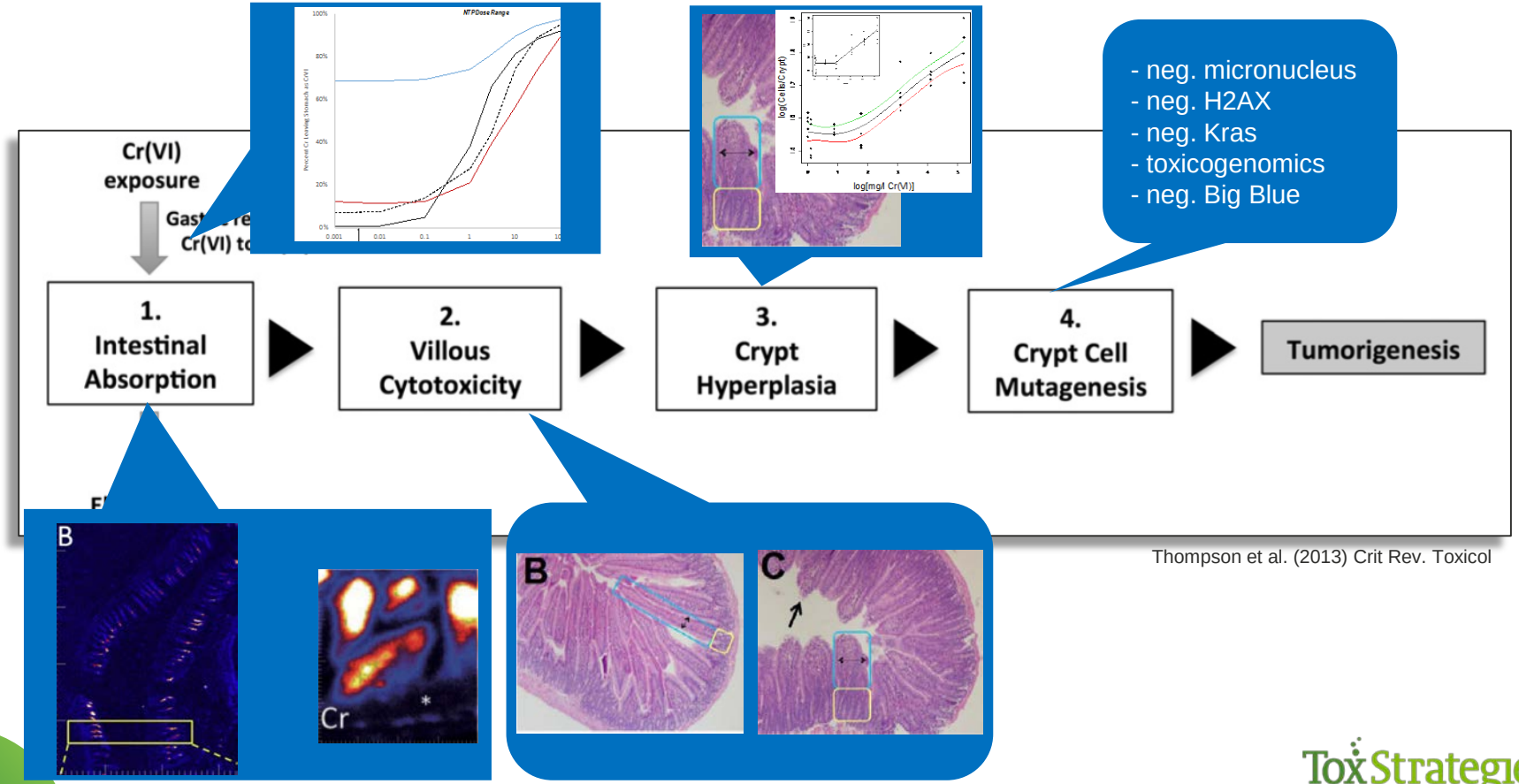
Non-mutagenic MOA for SI Tumors



Thompson et al. (2013) Crit Rev. Toxicol

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Outline

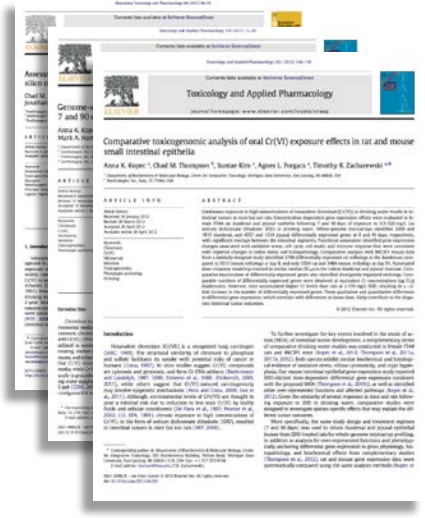
1. **Overview of MOA research project**
2. **Summary of proposed MOA for Cr(VI)**
3. **Open discussion on research findings**
    a) Q & A
    b) New MOA data
        i. Oral toxicogenomics
        ii. Histopathology
    c) New PK data
    d) Other topics
        i. Genotoxicity
        ii. Risk Assessment

Oral Toxicogenomics

Toxicogenomic Data

13 wk studies in mice and rats

SI omics



Oral mucosa omics = unpublished

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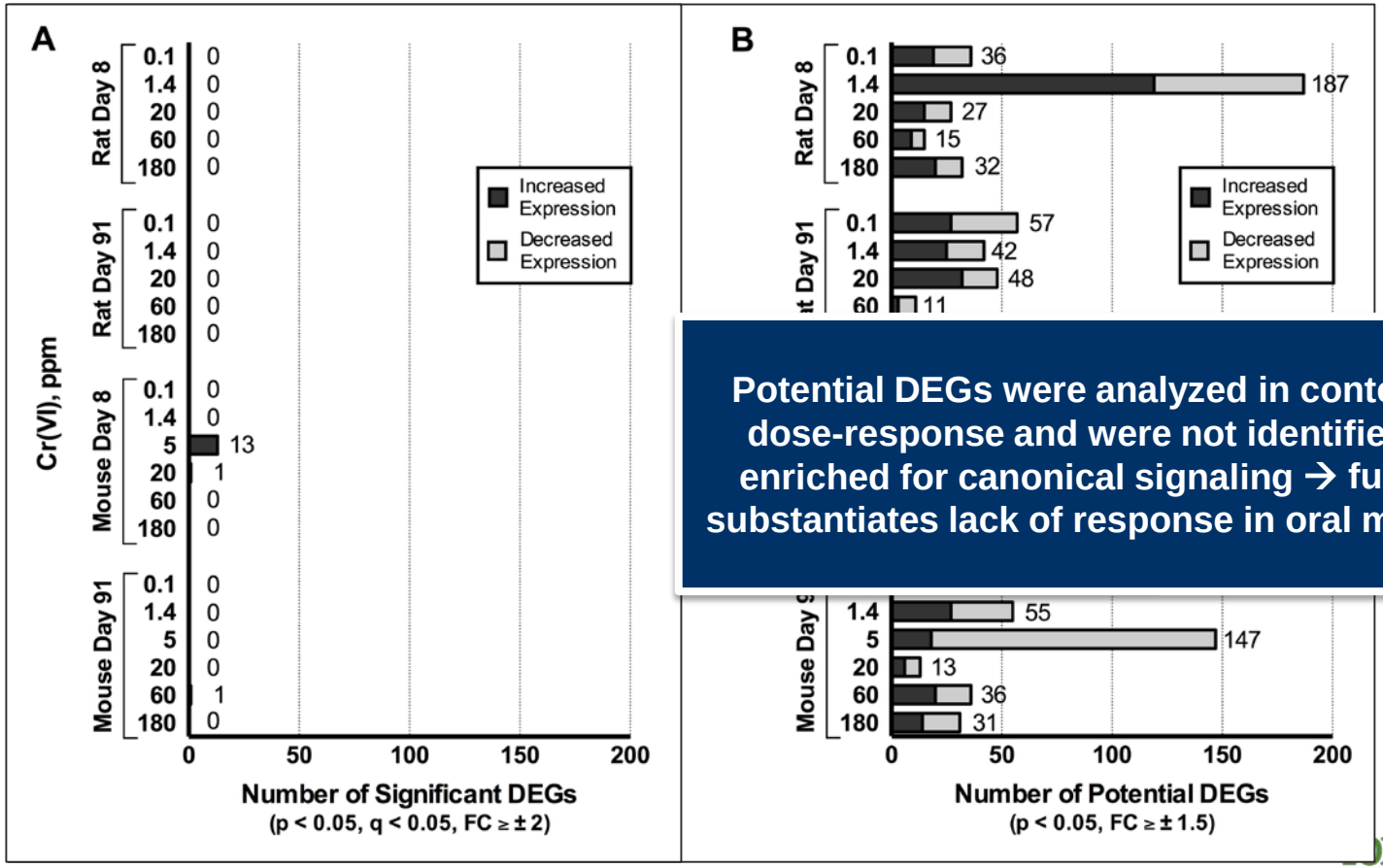
Updated Microarray Analyses: Differentially Expressed Genes

	0.3 mg/L	4 mg/L	14 mg/L	60 mg/L	170 mg/L	520 mg/L	All Doses
Oral Cavity							
Rat Day 8	0	0	N/A	0	0	0	0
Rat Day 91	0	0	N/A	0	0	0	0
Mouse Day 8	0	0	13	1	0	0	14
Mouse Day 91	0	0	0	0	1	0	1
Duo num							
Rat Day 8	0	3	N	233	823	629	913
Rat Day 91	0	0	N/A	18	64	118	136
Mouse							3029
Mouse							1099
Rat Day							1053
Rat Day							85
Mouse Day	22	19	11	202	737	1000	1371
Mouse Day 91	0	0	0	178	1366	1257	1563

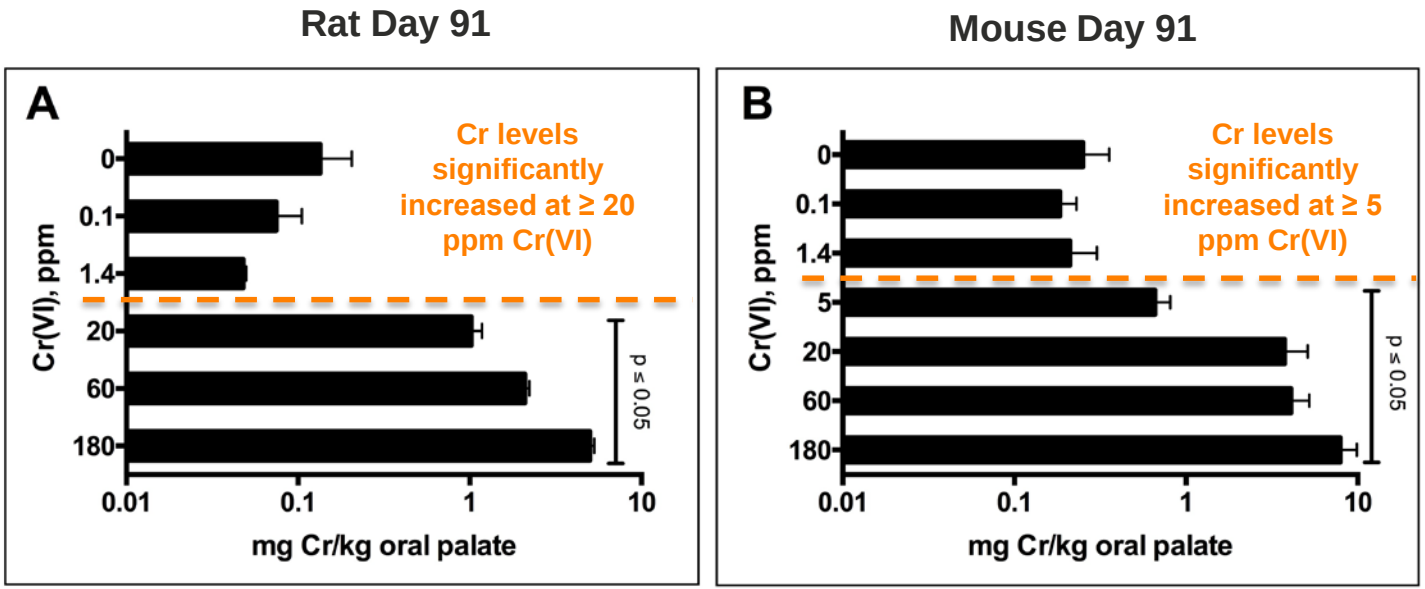
Oral cavity toxicogenomics have never been published…
Provide useful information for considering oral tumors...

Transcriptomic Responses in Oral Mucosa (Submitted)



Potential DEGs were analyzed in context of dose-response and were not identified as enriched for canonical signaling → further substantiates lack of response in oral mucosa

Tissue Dosimetry in Oral Mucosa



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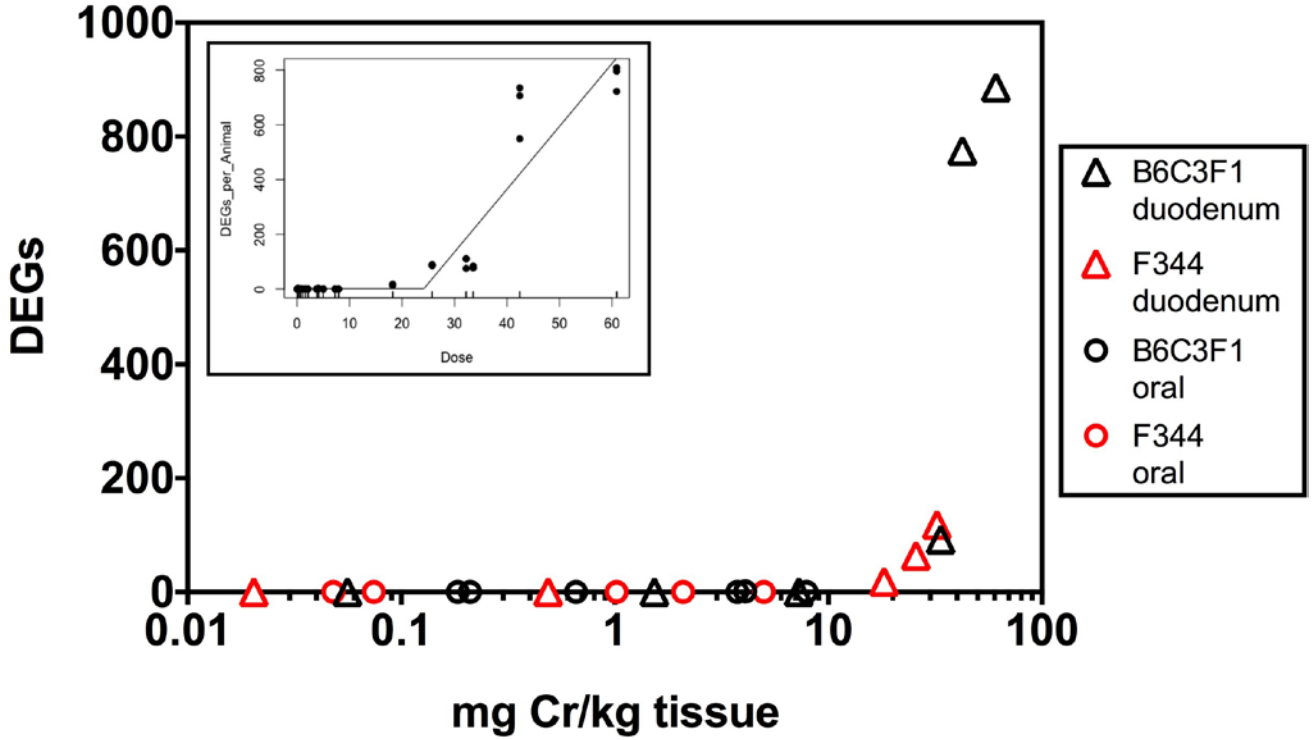
Tissue Dosimetry vs. DEGs after 90 Days of Cr(VI) Exposure



Transcriptomic responses occurred at > 10 mg/kg

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Lack of Transcript Changes is Consistent with Other Oral Mucosa Data

Summary of Effects in the Rat Oral Mucosa

Endpoint	Evidence
Histopathology	- No non-neoplastic or pre-neoplastic histopathological lesions have been detected in the rat oral mucosa following exposure to ≤180 ppm Cr(VI) for 7 days (Thompson et al., 2012), 13 weeks
Cr(VI) tissue absorption	
Mutation analysis	drinking water
Transcriptomic analyses	- No significant DEGs, or potentially altered pathways, associated with exposure to ≤180 ppm Cr(VI) in drinking water for 7 and 90 days

Data indicate that the oral tumors are not the result of direct action of Cr(VI) in oral mucosa

→ Findings are not compatible with linear MOA and linear low-dose extrapolation methods for setting toxicity criteria

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Histopathology

Non-neoplastic Lesions In NTP Study

Summary of the 2-Year Carcinogenesis Studies of Sodium Dichromate Dihydrate

	Male F344/N Rats	Female F344/N Rats	Male B6C3F1 Mice	Female B6C3F1 Mice
Concentrations in drinking water	0, 14.3, 57.3, 172, or 516 mg/L	0, 14.3, 57.3, 172, or 516 mg/L	0, 14.3, 28.6, 85.7, or 257.4 mg/L	0, 14.3, 57.3, 172, or 516 mg/L
Body weights	516 mg/L group less than the control group	516 mg/L group less than the control group	Exposed groups similar to control group	172 and 516 mg/L groups less than control group
Survival rates	28/50, 30/50, 30/49, 36/50, 29/49	33/50, 32/50, 32/50, 36/50, 31/50	33/50, 35/50, 35/50, 38/50, 32/50	37/50, 39/50, 45/50, 42/50, 42/50
Nonneoplastic effects	Liver: infiltration cellular, histiocyte (1/50, 0/50, 2/49, 5/50, 34/49) Small intestine, duodenum: infiltration cellular, histiocyte (0/48, 0/48, 6/47, 36/46, 47/48)	Liver: infiltration cellular, histiocyte (1/50, 5/50, 21/50, 42/50, 47/50) Small intestine, duodenum: infiltration cellular, histiocyte (0/46, 0/49, 1/48, 30/46, 47/50)	Small intestine, duodenum: epithelium, hyperplasia, diffuse (0/50, 11/50, 18/50, 42/50, 32/50); infiltration cellular, histiocyte (0/50, 2/50, 4/50, 37/50, 35/50) Lymph node, mesenteric:	Liver: infiltration cellular, histiocyte (2/49, 15/50, 23/50, 32/50, 45/50) Small intestine, duodenum: epithelium, hyperplasia, diffuse (0/50, 16/50, 35/50,

DEH was not specifically defined, but appeared to involve changes to both crypt and villus epithelium.

- In rats, the only non-neoplastic lesions in the Summary table were related to infiltration of histiocytes
- In mice, with one exception, all non-neoplastic lesions in the Summary table were related to infiltration of histiocytes
- Diffuse epithelial hyperplasia (DEH) occurred in the duodenum and jejunum (i.e. where SI tumors arose) of mice only
- 13-wk NTP studies also observed DEH in mice (but not rats)

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Intestinal Lesions in the MOA 13-wk Mouse Study

TOXICOLOGICAL SCIENCES 123(1), 58–70 (2011)
doi:10.1093/toxsci/kfr164
Advance Access publication June 28, 2011

Investigation of the Mode of Action Underlying the Tumorigenic Response Induced in B6C3F1 Mice Exposed Orally to Hexavalent Chromium

Chad M. Thompson,*[,1] Deborah M. Proctor,† Laurie C. Haws,‡ Charles D. Hébert,§ Sheila D. Grimes,§ Howard G. Shertzer,¶ Anna K. Kopec,|| J.Gregory Hixon,‡ Timothy R. Zacharewski,|| and Mark A. Harris*

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Received April 22, 2011; accepted June 14, 2011

Chronic ingestion of high concentrations of hexavalent chromium [Cr(VI)] in drinking water induces intestinal tumors in mice. To investigate the mode of action (MOA) underlying these tumors, a 90-day drinking water study was conducted using similar exposure conditions as in a previous cancer bioassay, as well as lower (heretofore unexamined) drinking water concentrations. Tissue samples were collected in mice exposed for 7 or 90 days and subjected to histopathological, biochemical, toxicogenomic, and toxicokinetic analyses. Described herein are the results of toxicokinetic, biochemical, and pathological findings. Following 90 days of exposure to 0.3–520 mg/l of sodium dichromate dihydrate (SDD), total chromium concentrations in the duodenum were significantly elevated at ≥ 14 mg/l. At these concentrations, significant decreases in the reduced-to-oxidized glutathione ratio (GSH/GSSG) were observed. Beginning at 60 mg/l, intestinal lesions were observed including villous cytoplasmic vacuolization. Atrophy, apoptosis, and crypt hyperplasia were evident at ≥ 170 mg/l. Protein carbonyls were elevated at concentrations ≥ 4 mg/l SDD, whereas oxidative DNA damage, as assessed by 8-hydroxydeoxyguanosine, was not increased in any treatment group. Significant decreases in the GSH/GSSG ratio and similar histopathological lesions as observed in the duodenum were also observed in the jejunum following 90 days of exposure. Cytokine levels (e.g., interleukin-1β) were generally depressed or unaltered at the termination of the study. Overall, the data suggest that Cr(VI) in drinking water can induce oxidative stress, villous cytotoxicity, and crypt hyperplasia in the mouse intestine and may underlie the MOA of intestinal carcinogenesis in mice.

Key Words: **risk assessment; carcinogenesis; hexavalent chromium; Cr(VI); mode of action; MOA.**

Until recently, there was inadequate information to assess the oral carcinogenicity of hexavalent chromium [Cr(VI)] (International Agency for Research on Cancer, 1990; U.S. EPA, 1991, 1998); however, a 2-year cancer bioassay conducted by the National Toxicology Program (NTP) reported that administration of Cr(VI) in drinking water (in the form of sodium dichromate dihydrate [SDD]) induced tumors in the small intestines of mice at ≥ 57 mg/l SDD (≥ 20 mg/l Cr(VI)) and in the oral cavities of rats at ≥ 172 mg/l SDD (≥ 60 mg/l Cr(VI)) (NTP, 2008; Stout *et al.*, 2009). Because low levels of Cr(VI) are prevalent in groundwater in certain geographical areas (Oze *et al.*, 2007), wide-spread impact to some drinking water supplies has occurred. For example, approximately one third of California drinking water supplies contain low levels of Cr(VI)—mostly ranging from 0.001 to 0.005 mg/l (California Department of Health Services, 2009); thus, the effects of Cr(VI) at low concentrations is of interest. Notably, the concentrations inducing cancer in rodents are orders of magnitude greater than typical drinking water exposures (Thompson *et al.*, 2011).

In the NTP 2-year cancer bioassay (NTP, 2008), diffuse intestinal epithelial hyperplasia was observed in the small intestine of mice at all concentrations examined (NTP, 2008) and at ≥ 62.5 mg/l SDD in an earlier 90-day study (NTP, 2007). In stark contrast to mice, neither diffuse hyperplasia nor tumors were reported in the rat small intestine at any of the drinking water concentrations tested (NTP, 2007, 2008). The non-neoplastic intestinal lesions in the mouse were characterized by the NTP investigators as "regenerative hyperplasia secondary to previous epithelial cell injury" (NTP, 2008) and suggest that the tumors may have been caused by prolonged tissue injury and proliferative pressure on crypt cells (Thompson *et al.*, 2011). Despite evidence that chromium can be genotoxic and mutagenic (McCarroll *et al.*, 2010; Nickens *et al.*, 2010; O'Brien *et al.*, 2003), there are limited data to evaluate the mode of action (MOA) of Cr(VI) in the small intestine. Thus, the MOA for small intestinal tumors at high concentration exposures is uncertain,

Southern Research pathologists did not use term DEH, but specified effects in crypt or villus!

This was more informative, but raised questions on how to directly compare to NTP results!

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Intestinal Lesions in MOA 13-wk Rat Study

TOXICOLOGICAL SCIENCES 125(1), 79–90 (2012)
doi:10.1093/toxsci/kfr280
Advance Access publication October 19, 2011

Comparison of the Effects of Hexavalent Chromium in the Alimentary Canal of F344 Rats and B6C3F1 Mice Following Exposure in Drinking Water: Implications for Carcinogenic Modes of Action

Chad M. Thompson,*,[1] Deborah M. Proctor,† Mina Suh,† Laurie C. Haws,‡ Charles D. Hébert,§ Jill F. Mar
Howard G. Shertzer,¶ J. Gregory Hixon,‡ and Mark A. Harris*

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Received August 22, 2011; accepted October 10, 2011

Exposure to high concentrations of hexavalent chromium (Cr[VI]) in drinking water is reported to induce oral mucosa tumors in F344 rats and intestinal tumors in B6C3F1 mice. To investigate the modes of action underlying these tumors, 90-day drinking water studies (with interim necropsy at day 8) were conducted with concentrations of 0.1–182 mg/l Cr(VI), administered as 0.3–520 mg/l sodium dichromate dihydrate. Blood and tissue samples were analyzed for chromium content, oxidative stress, iron levels, and gross and microscopic lesions. Results for the F344 rats are described herein and compared with results from B6C3F1 mice published previously. After 90 days of exposure, total chromium concentrations in the rat and mouse oral mucosae were comparable, yet significant dose-dependent decreases in the reduced-to-oxidized glutathione ratio (GSH/GSSG) were observed only in rats. In the duodenum, changes in GSH/GSSG were only observed in mice. Levels of 8-hydroxydeoxyguanosine were not increased in the oral or duodenal mucosae of either species. Glutathione levels were increased in the duodenum but decreased in the jejunum of both species, indicating potential differential responses in the intestinal segments. Histiocytic infiltration was observed in the duodenum of both species, yet duodenal cytokines were repressed in mice but increased in rats. Serum and bone marrow iron levels were more decreased in rats than mice. Collectively, these data suggest that Cr(VI)-induced carcinogenesis in the rodent alimentary canal involves oxidative stress; however, differences in histopathology, cytokines, and iron status suggest potential contributions from other factors as well.

Key Words: drinking water; oxidative stress; carcinogenesis; Cr(VI); MOA.

Chronic ingestion of hexavalent chromium (Cr(VI)), in the form of sodium dichromate dihydrate ($Na_2Cr_2O_7 \cdot 2H_2O$ or SDD), in drinking water has been found to induce oral mucosa tumors in rats at concentrations ≥ 172 mg SDD/l, and intestinal tumors in mice at concentrations ≥ 57.3 mg SDD/l (National Toxicology Program [NTP], 2008; Stout *et al.*, 2009). Villous cytotoxicity and crypt hyperplasia were observed in the mouse small intestine, whereas no obvious non-neoplastic lesions were observed in the oral mucosae of rats (NTP, 2008; Stout *et al.*, 2009). The different lesions suggest that the tumors in the two sites may have arisen through different carcinogenic modes of action (MOAs). Although the NTP (2008) 2-year bioassay demonstrated that Cr(VI) was carcinogenic in rodents following chronic exposure to high concentrations in drinking water, the study did not provide adequate data for understanding how the tumors arose or why different tumors were observed in each species. Because mice and rats developed tumors in different tissues of the alimentary canal, comparisons of species-specific pathology and biochemistry in the target tissues (i.e., oral and intestinal mucosae) are expected to inform the MOAs for carcinogenicity in each tissue.

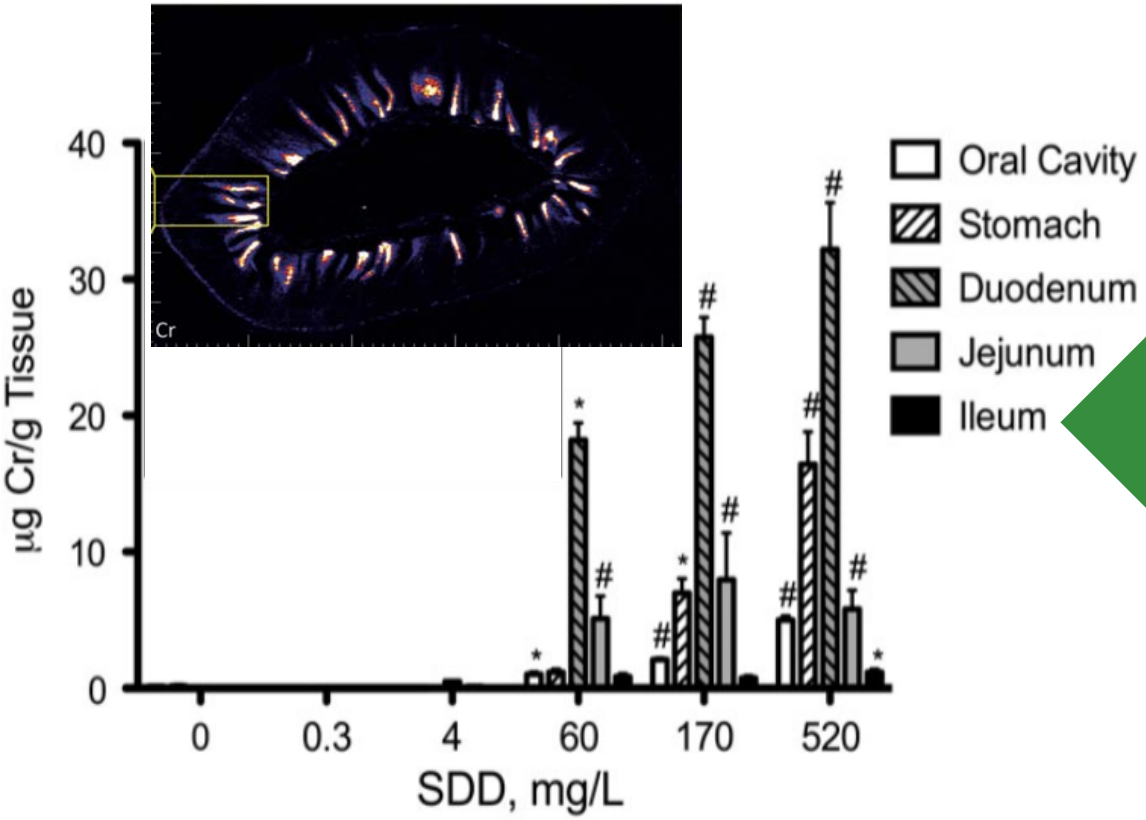
Several authors have proposed or discussed possible MOAs for alimentary cancers in rodents (McCarroll *et al.*, 2010; Stern, 2010; Thompson *et al.*, 2011a). However, currently available data are insufficient to conclusively support any of the hypothesized MOAs. This is especially true for oral squamous cell carcinomas originating from the rat palate after 2 years of exposure to SDD, where no non-neoplastic lesions were reported (NTP, 2007, 2008; Stout *et al.*, 2009). The number of oral cavity tumors was significantly elevated as compared to current and historical controls in the highest dose group (516 mg/l) in both males (6/49) and females (11/50), as well as historical controls in females in the 172 mg/l treatment group (2/50) (NTP, 2008; Stout *et al.*, 2009). Stout *et al.* (2009) noted that 21 chemicals have been shown to cause oral cavity tumors in rats, but no chemicals have been shown to cause oral cavity tumors in male mice, and only one caused oral cavity tumors in female mice. These findings may suggest an inherent susceptibility of rats to oral cancers.

Southern Research pathologists saw effects in rat small intestine!

Initial explanations:
1. inter-study variability
2. water/Cr6 consumption differences

This led to some questioning the relevance of MOA studies…

Cr also Highest in Duodenum and Villi of Rats



Began to suspect mild lesions may have been overlooked in NTP rats.

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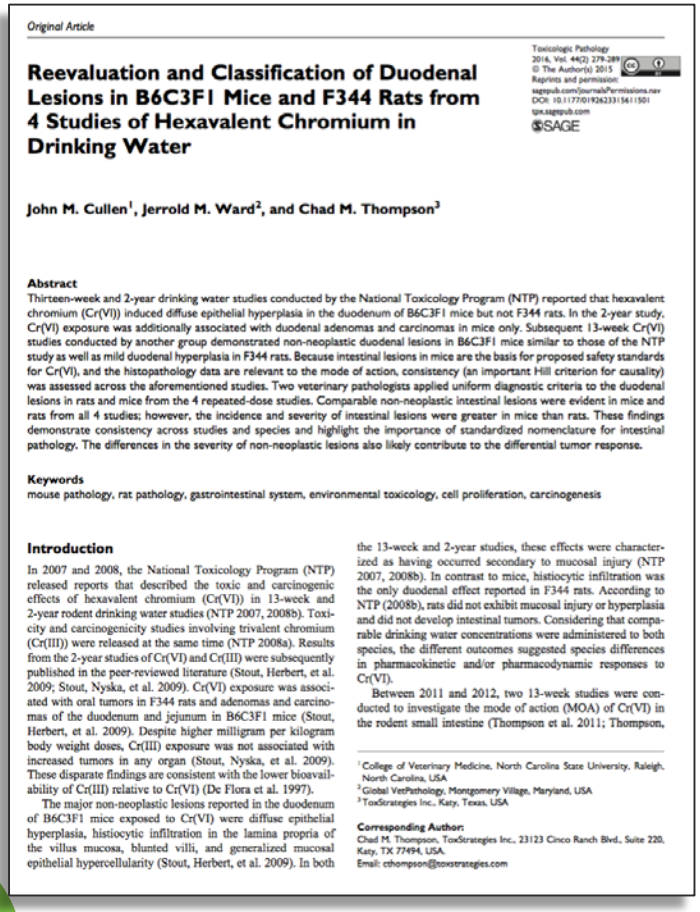
Re-evaluation of MOA and NTP H&E Stained Tissue Sections



Original Article

Reevaluation and Classification of Duodenal Lesions in B6C3F1 Mice and F344 Rats from 4 Studies of Hexavalent Chromium in Drinking Water

Toxicologic Pathology
2016, Vol. 44(2) 279-289

DOI: 10.1177/0192623315611501
tpx.sagepub.com
SAGE

John M. Cullen[1], Jerrold M. Ward[2], and Chad M. Thompson[3]

Abstract

Thirteen-week and 2-year drinking water studies conducted by the National Toxicology Program (NTP) reported that hexavalent chromium (Cr(VI)) induced diffuse epithelial hyperplasia in the duodenum of B6C3F1 mice but not F344 rats. In the 2-year study, Cr(VI) exposure was additionally associated with duodenal adenomas and carcinomas in mice only. Subsequent 13-week Cr(VI) studies conducted by another group demonstrated non-neoplastic duodenal lesions in B6C3F1 mice similar to those of the NTP study as well as mild duodenal hyperplasia in F344 rats. Because intestinal lesions in mice are the basis for proposed safety standards for Cr(VI), and the histopathology data are relevant to the mode of action, consistency (an important Hill criterion for causality) was assessed across the aforementioned studies. Two veterinary pathologists applied uniform diagnostic criteria to the duodenal lesions in rats and mice from the 4 repeated-dose studies. Comparable non-neoplastic intestinal lesions were evident in mice and rats from all 4 studies; however, the incidence and severity of intestinal lesions were greater in mice than rats. These findings demonstrate consistency across studies and species and highlight the importance of standardized nomenclature for intestinal pathology. The differences in the severity of non-neoplastic lesions also likely contribute to the differential tumor response.

Keywords

mouse pathology, rat pathology, gastrointestinal system, environmental toxicology, cell proliferation, carcinogenesis

Introduction

In 2007 and 2008, the National Toxicology Program (NTP) released reports that described the toxic and carcinogenic effects of hexavalent chromium (Cr(VI)) in 13-week and 2-year rodent drinking water studies (NTP 2007, 2008b). Toxicity and carcinogenicity studies involving trivalent chromium (Cr(III)) were released at the same time (NTP 2008a). Results from the 2-year studies of Cr(VI) and Cr(III) were subsequently published in the peer-reviewed literature (Stout, Herbert, et al. 2009; Stout, Nyska, et al. 2009). Cr(VI) exposure was associated with oral tumors in F344 rats and adenomas and carcinomas of the duodenum and jejunum in B6C3F1 mice (Stout, Herbert, et al. 2009). Despite higher milligram per kilogram body weight doses, Cr(III) exposure was not associated with increased tumors in any organ (Stout, Nyska, et al. 2009). These disparate findings are consistent with the lower bioavailability of Cr(III) relative to Cr(VI) (De Flora et al. 1997).

The major non-neoplastic lesions reported in the duodenum of B6C3F1 mice exposed to Cr(VI) were diffuse epithelial hyperplasia, histiocytic infiltration in the lamina propria of the villus mucosa, blunted villi, and generalized mucosal epithelial hypercellularity (Stout, Herbert, et al. 2009). In both the 13-week and 2-year studies, these effects were characterized as having occurred secondary to mucosal injury (NTP 2007, 2008b). In contrast to mice, histiocytic infiltration was the only duodenal effect reported in F344 rats. According to NTP (2008b), rats did not exhibit mucosal injury or hyperplasia and did not develop intestinal tumors. Considering that comparable drinking water concentrations were administered to both species, the different outcomes suggested species differences in pharmacokinetic and/or pharmacodynamic responses to Cr(VI).

Between 2011 and 2012, two 13-week studies were conducted to investigate the mode of action (MOA) of Cr(VI) in the rodent small intestine (Thompson et al. 2011; Thompson,

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Email: cthompson@toxstrategies.com

Diagnostic Criteria	Description
Villus, histiocytic cellular infiltrates	primarily in the lamina propria of the villus tips, and characterized by small nodular aggregations of macrophages with abundant, faintly granular, eosinophilic cytoplasm
Villus, atrophy/blunting	villi appeared shortened and/or thickened relative to those of control animals
Villus, enterocyte vacuolation	single or multiple, sharply defined, clear or slightly flocculent spaces within the cytoplasm of terminal villus enterocytes
Villus, single cell necrosis	primarily in the villus tips either as shrunken cells with densely eosinophilic cytoplasm and pyknotic or fragmented nuclei, or as cells with karyorrhectic nuclei
Crypt, epithelial hyperplasia	elongated crypts that were lined by increased numbers of crowded columnar enterocytes with hyperchromatic basophilic cytoplasm and nuclear chromatin clumping. In more extensively affected cases (mice especially), the hyperplastic enterocytes additionally displayed increased cell height and tinctorial changes compared to those of controls

Grade	
1	Minimal
2	Mild
3	Moderate

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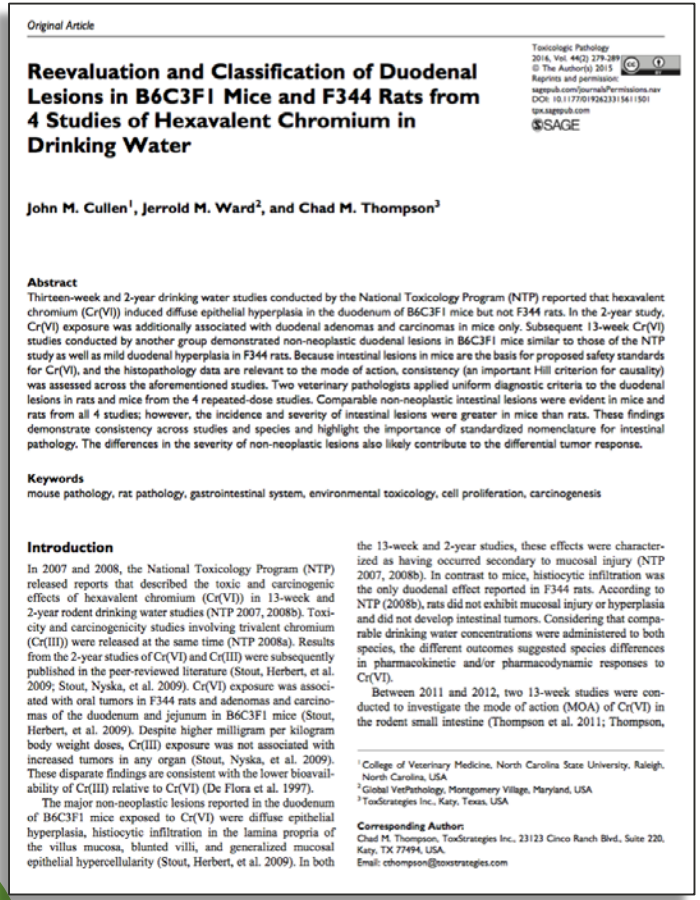
Conclusion 1: Mice in NTP and MOA 13-wk Studies had Similar Effects at Comparable Doses (mg/kg SDD)



Original Article

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Reevaluation and Classification of Duodenal Lesions in B6C3F1 Mice and F344 Rats from 4 Studies of Hexavalent Chromium in Drinking Water

John M. Cullen[1], Jerrold M. Ward[2], and Chad M. Thompson[3]

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Thirteen-week and 2-year drinking water studies conducted by the National Toxicology Program (NTP) reported that hexavalent chromium (Cr(VI)) induced diffuse epithelial hyperplasia in the duodenum of B6C3F1 mice but not F344 rats. In the 2-year study, Cr(VI) exposure was additionally associated with duodenal adenomas and carcinomas in mice only. Subsequent 13-week Cr(VI) studies conducted by another group demonstrated non-neoplastic duodenal lesions in B6C3F1 mice similar to those of the NTP study as well as mild duodenal hyperplasia in F344 rats. Because intestinal lesions in mice are the basis for proposed safety standards for Cr(VI), and the histopathology data are relevant to the mode of action, consistency (an important Hill criterion for causality) was assessed across the aforementioned studies. Two veterinary pathologists applied uniform diagnostic criteria to the duodenal lesions in rats and mice from the 4 repeated-dose studies. Comparable non-neoplastic intestinal lesions were evident in mice and rats from all 4 studies; however, the incidence and severity of intestinal lesions were greater in mice than rats. These findings demonstrate consistency across studies and species and highlight the importance of standardized nomenclature for intestinal pathology. The differences in the severity of non-neoplastic lesions also likely contribute to the differential tumor response.

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Between 2011 and 2012, two 13-week studies were conducted to investigate the mode of action (MOA) of Cr(VI) in the rodent small intestine (Thompson et al. 2011; Thompson,

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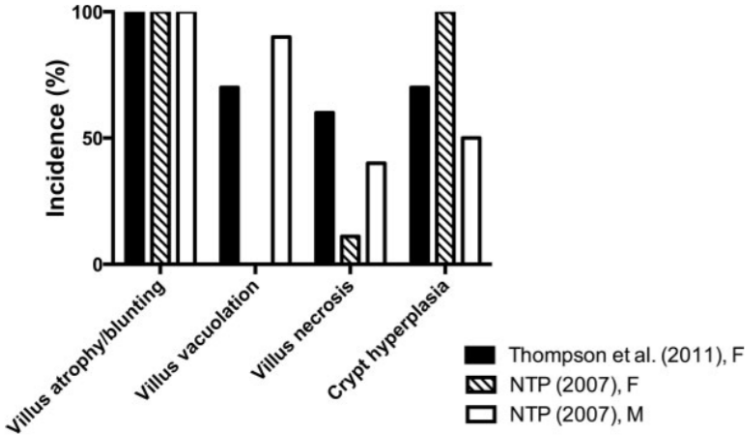
13-wk studies



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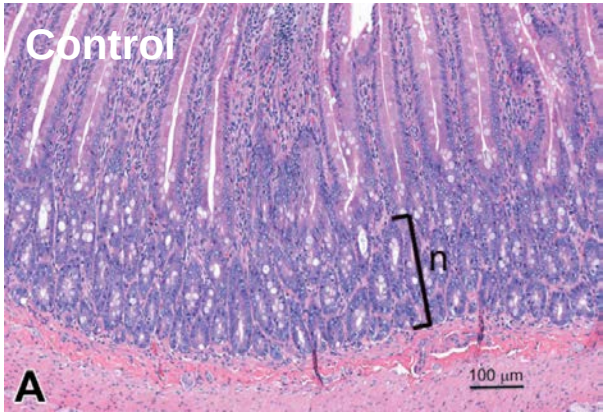
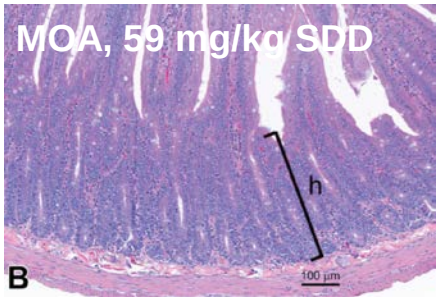
Conclusion 2: Rats in NTP and MOA 13-wk Studies had Similar Effects at Comparable Doses (mg/kg SDD)





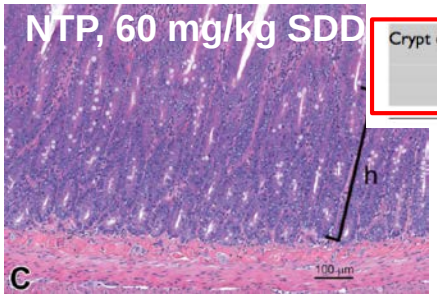


Table 3. Reevaluation of Duodenal Lesions in F344 Rats in the 13-week Drinking Water Studies.

Study		Thompson et al. (2012), female			NTP (2007), female		NTP (2007), male		
Nominal concentration milligram per liter SDD		0	170	520	0	1,000	0	500	1,000
Nominal dose milligram per kilogram SDD		0	20	59	0	61	0	32	60
Number of rats examined		10	10	10	10	10	10	10	10
Villus histiocytic cellular infiltrates	Grade 1	0[a]	9	5	1	7	0	4	6
	Grade 2	0	1	4	0	2	0	1	4
	Grade 3	0	0	0	0	0	0	0	0
	All grades	0	10	9	1	9	0	5	10
Villus atrophy/blunting	Grade 1	0	4	8	0	0	0	0	0
	Grade 2	0	0	0	0	0	0	0	0
	Grade 3	0	0	0	0	0	0	0	0
	All grades	0	4	8	0	0	0	0	0
Villus enterocyte vacuolation	Grade 1	0	0	0	0	0	0	0	0
	Grade 2	0	0	0	0	0	0	0	0
	Grade 3	0	0	0	0	0	0	0	0
	All grades	0	0	0	0	0	0	0	0
Villus single-cell necrosis	Grade 1	0	0	3	0	0	0	0	1
	Grade 2	0	0	0	0	0	0	0	0
	Grade 3	0	0	0	0	0	0	0	0
	All grades	0	0	3	0	0	0	0	1
Crypt epithelial hyperplasia	Grade 1	0	3	2	0	3	0	5	6
	Grade 2	0	0	0	0	0	0	0	1
	Grade 3	0	0	0	0	0	0	0	0
	All grades	0	3	2	0	3	0	5	7

Source: Cullen et al. (2015) *Toxicologic Pathology*

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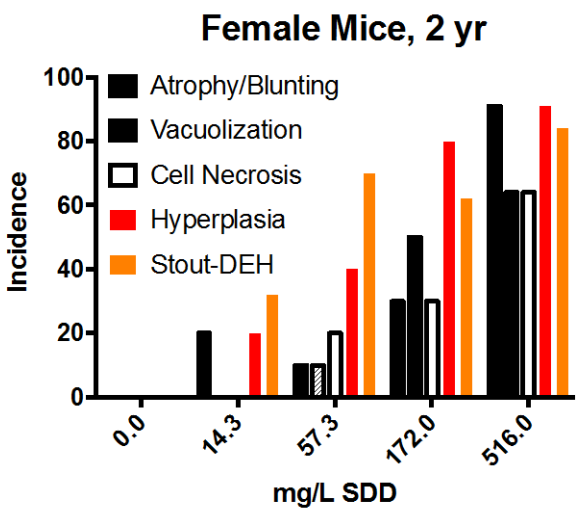
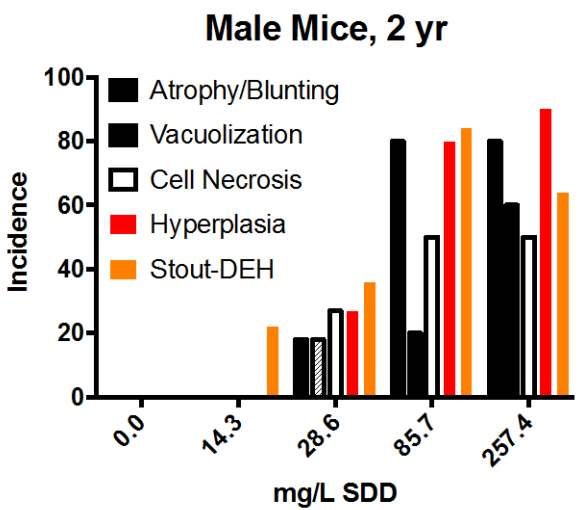
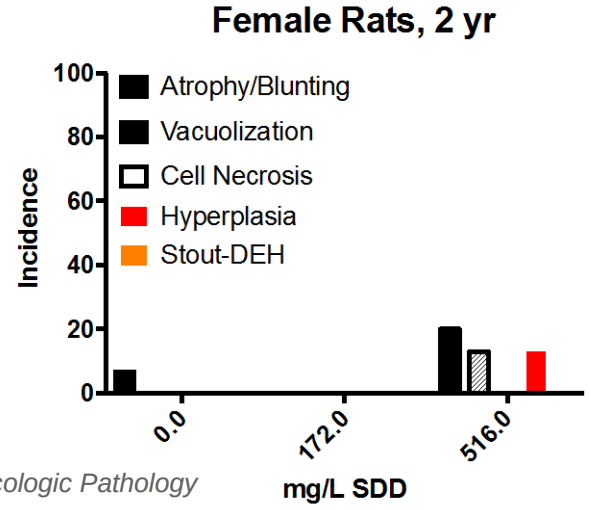
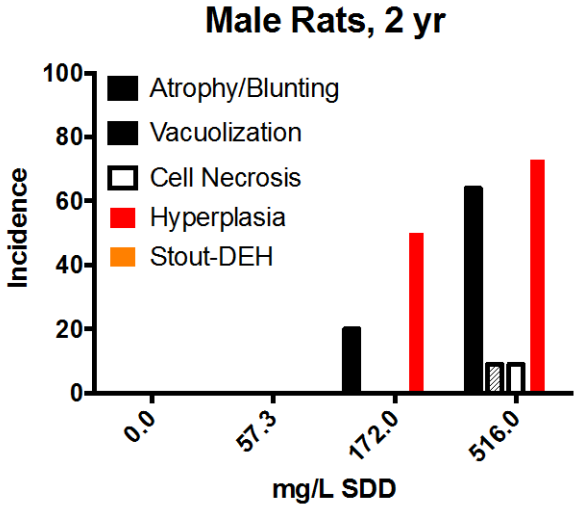
Re-evaluation of 2-yr NTP Data:



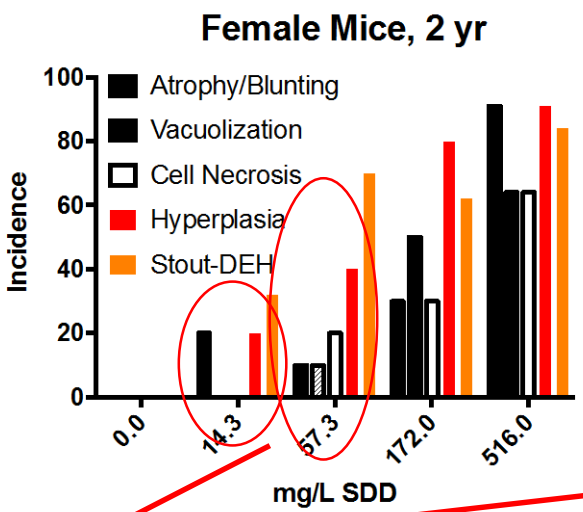


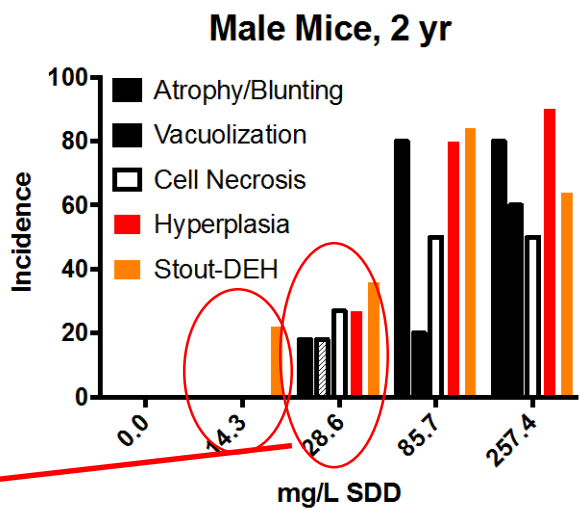




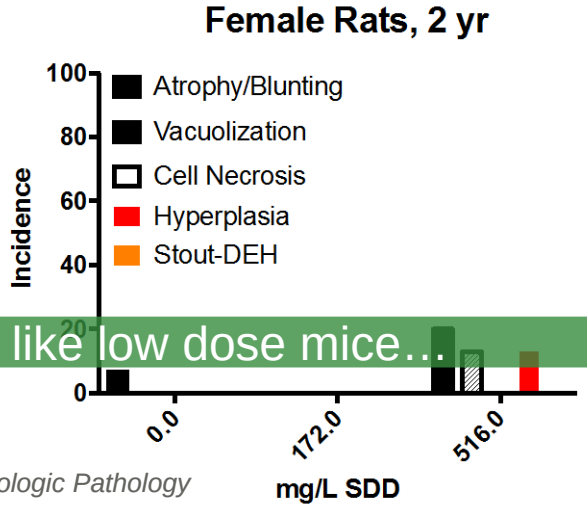
Source: Cullen et al. (2015) *Toxicologic Pathology*

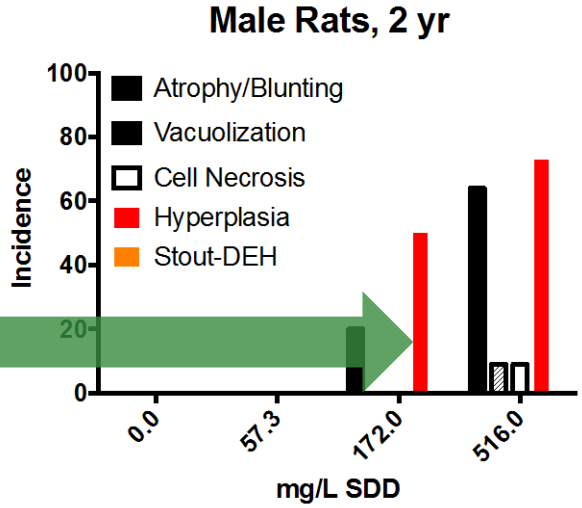
Re-evaluation of 2-yr NTP Data:





Note that these were not carcinogenic (relative to concurrent controls).

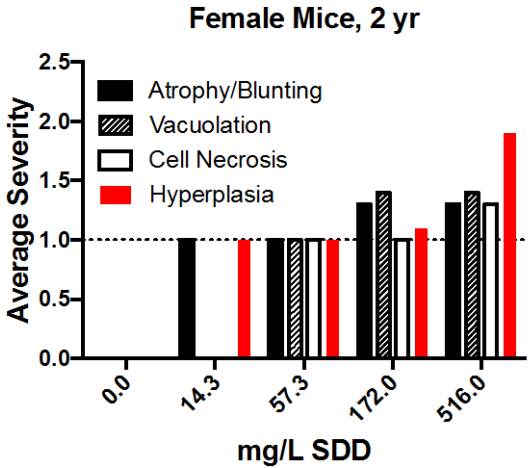


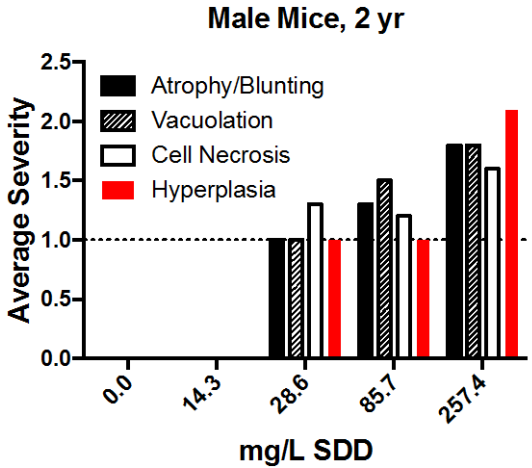


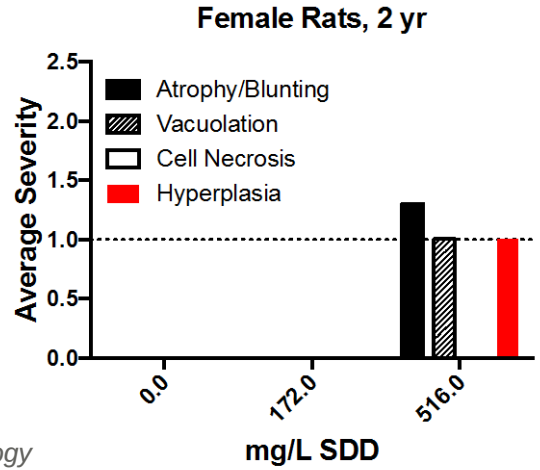
High dose rats are like low dose mice…

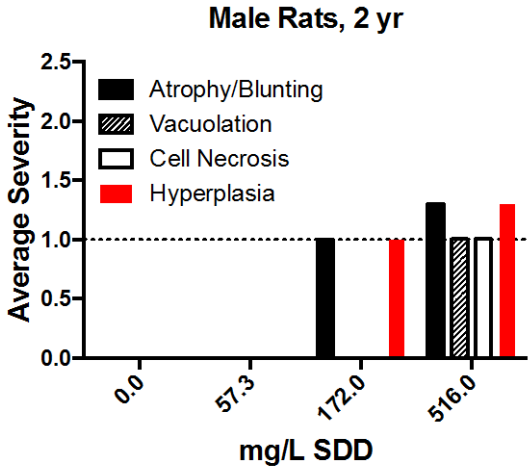
Source: Cullen et al. (2015) *Toxicologic Pathology*

Re-evaluation of 2-yr NTP Data:



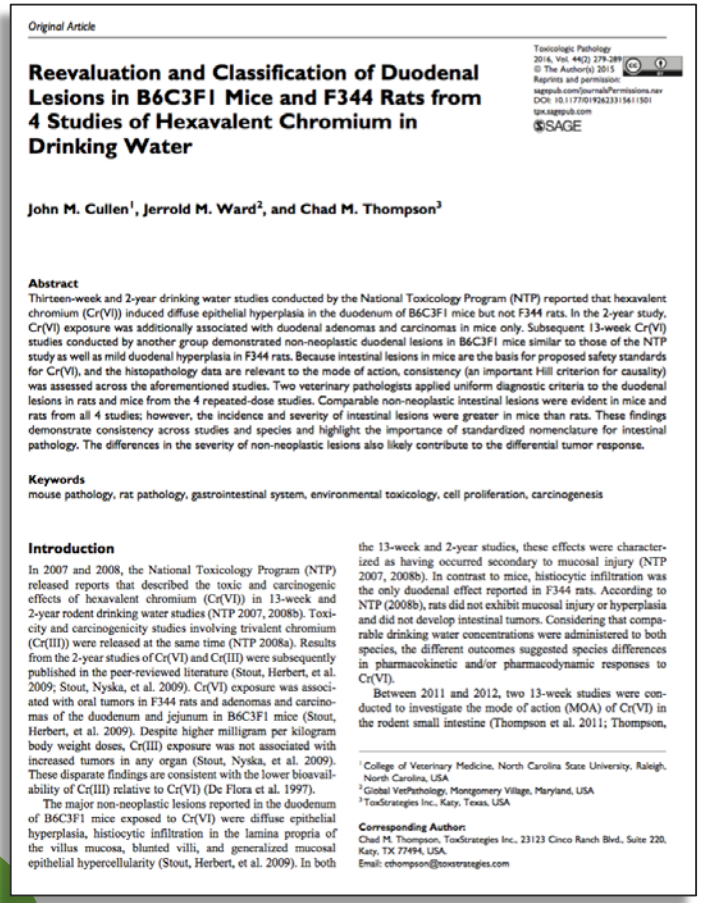






Source: Cullen et al. (2015) *Toxicologic Pathology*

Re-evaluation of Tissue Sections: Final Conclusions



Original Article

Reevaluation and Classification of Duodenal Lesions in B6C3F1 Mice and F344 Rats from 4 Studies of Hexavalent Chromium in Drinking Water

Toxicologic Pathology
2016, Vol. 44(2) 279-289

DOI: 10.1177/0192623315611501
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John M. Cullen[1], Jerrold M. Ward[2], and Chad M. Thompson[3]

Abstract

Thirteen-week and 2-year drinking water studies conducted by the National Toxicology Program (NTP) reported that hexavalent chromium (Cr(VI)) induced diffuse epithelial hyperplasia in the duodenum of B6C3F1 mice but not F344 rats. In the 2-year study, Cr(VI) exposure was additionally associated with duodenal adenomas and carcinomas in mice only. Subsequent 13-week Cr(VI) studies conducted by another group demonstrated non-neoplastic duodenal lesions in B6C3F1 mice similar to those of the NTP study as well as mild duodenal hyperplasia in F344 rats. Because intestinal lesions in mice are the basis for proposed safety standards for Cr(VI), and the histopathology data are relevant to the mode of action, consistency (an important Hill criterion for causality) was assessed across the aforementioned studies. Two veterinary pathologists applied uniform diagnostic criteria to the duodenal lesions in rats and mice from the 4 repeated-dose studies. Comparable non-neoplastic intestinal lesions were evident in mice and rats from all 4 studies; however, the incidence and severity of intestinal lesions were greater in mice than rats. These findings demonstrate consistency across studies and species and highlight the importance of standardized nomenclature for intestinal pathology. The differences in the severity of non-neoplastic lesions also likely contribute to the differential tumor response.

Keywords

mouse pathology, rat pathology, gastrointestinal system, environmental toxicology, cell proliferation, carcinogenesis

Introduction

In 2007 and 2008, the National Toxicology Program (NTP) released reports that described the toxic and carcinogenic effects of hexavalent chromium (Cr(VI)) in 13-week and 2-year rodent drinking water studies (NTP 2007, 2008b). Toxicity and carcinogenicity studies involving trivalent chromium (Cr(III)) were released at the same time (NTP 2008a). Results from the 2-year studies of Cr(VI) and Cr(III) were subsequently published in the peer-reviewed literature (Stout, Herbert, et al. 2009; Stout, Nyska, et al. 2009). Cr(VI) exposure was associated with oral tumors in F344 rats and adenomas and carcinomas of the duodenum and jejunum in B6C3F1 mice (Stout, Herbert, et al. 2009). Despite higher milligram per kilogram body weight doses, Cr(III) exposure was not associated with increased tumors in any organ (Stout, Nyska, et al. 2009). These disparate findings are consistent with the lower bioavailability of Cr(III) relative to Cr(VI) (De Flora et al. 1997).

The major non-neoplastic lesions reported in the duodenum of B6C3F1 mice exposed to Cr(VI) were diffuse epithelial hyperplasia, histiocytic infiltration in the lamina propria of the villus mucosa, blunted villi, and generalized mucosal epithelial hypercellularity (Stout, Herbert, et al. 2009). In both the 13-week and 2-year studies, these effects were characterized as having occurred secondary to mucosal injury (NTP 2007, 2008b). In contrast to mice, histiocytic infiltration was the only duodenal effect reported in F344 rats. According to NTP (2008b), rats did not exhibit mucosal injury or hyperplasia and did not develop intestinal tumors. Considering that comparable drinking water concentrations were administered to both species, the different outcomes suggested species differences in pharmacokinetic and/or pharmacodynamic responses to Cr(VI).

Between 2011 and 2012, two 13-week studies were conducted to investigate the mode of action (MOA) of Cr(VI) in the rodent small intestine (Thompson et al. 2011; Thompson,

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- "In summary, this review demonstrated that the non-neoplastic histopathologic effects of Cr(VI) in the intestines of mice and rats of the NTP (2007, 2008b) and [MOA] studies were all qualitatively similar, which suggests that the findings were pathogenically interrelated."
- "Specifically, villus atrophy/blunting, enterocyte vacuolation, single-cell necrosis, and crypt epithelial hyperplasia portray a process in which chemically induced villus enterocyte cytotoxicity resulted in regenerative crypt epithelial hyperplasia."
- "This sequela of events, which were more prevalent and severe in mice than rats, could have contributed to the development of duodenal neoplasms in mice."

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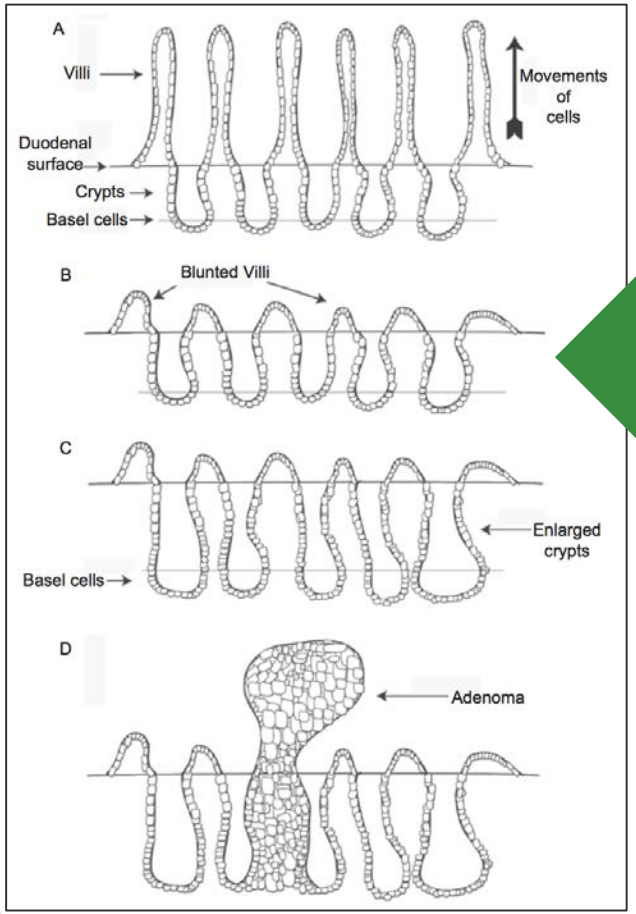
Relationship Between Villous Cytotoxicity & Crypt Proliferation



Source: Cohen et al. (2010) Crit Rev Toxicol 40: 531

This is a cartoon! The tissue does not wait to respond. Villi do not visibly blunt *before* crypt begins to respond.

When injury is mild, increased activity of the crypt replication zone compensates for the increased cell loss and there is [illegible] although the [illegible]ngth and in [illegible]re and more [illegible]eases further, enterocytes migrating upwards to cover villi do not have sufficient time to mature and tend to remain crowded together. The villi become shorter and broader…

(Brown, 2013, *Morson and Dawson's Gastrointestinal Pathology*)

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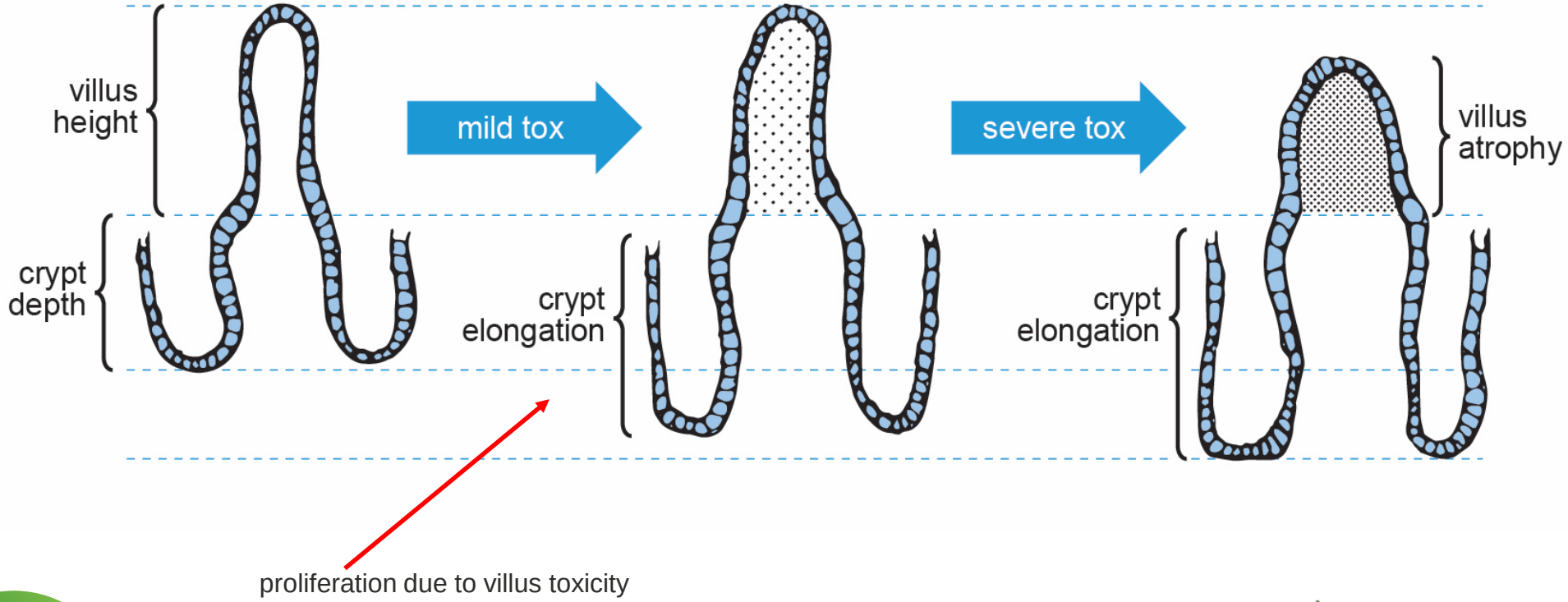
Revised Cartoon for MOA



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Pharmacokinetics

Update of PBPK Modeling Activities for Hexavalent Cr

Meeting with USEPA
August 10, 2016
Chris Kirman

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Overview for Pharmacokinetics

- Background
- New PK Data
- Refinements to PBPK model
- PK Publications in 2016

Background

Objective of PBPK Modeling for CrVI:

- Simulate rodents exposed to CrVI under conditions of the NTP cancer bioassay (NTP, 2008)
- Support risk assessment decisions regarding human populations exposed to CrVI
  - *Target tissue dosimetry (mouse small intestines)*
  - *Interspecies extrapolation*
  - *High-to-low dose extrapolation*
  - *Address variation and sensitive subpopulations*

Background (cont'd)

Recent PK/Modeling Publications for Chromium

- Ex Vivo Studies on Reduction of CrVI by Gastric Fluid
  - *Rats and Mice: Proctor et al. (2012)*
  - *Humans: Kirman et al. (2013a)*
  - *Refinement: Schlosser and Sasso (2014)*
- Physiologically Based Pharmacokinetic (PBPK) Model Development
  - *Rats and Mice: Kirman et al. (2013b)*
  - *Humans: Kirman et al. (2013a)*
  - *Evaluation: Sasso and Schlosser (2015)*
    - Results for EPA and our model versions are very similar
- PBPK Model Application
  - *RfD Derivation: Thompson et al. (2014)*

Background (cont'd)

Data gaps and limitations identified previously:

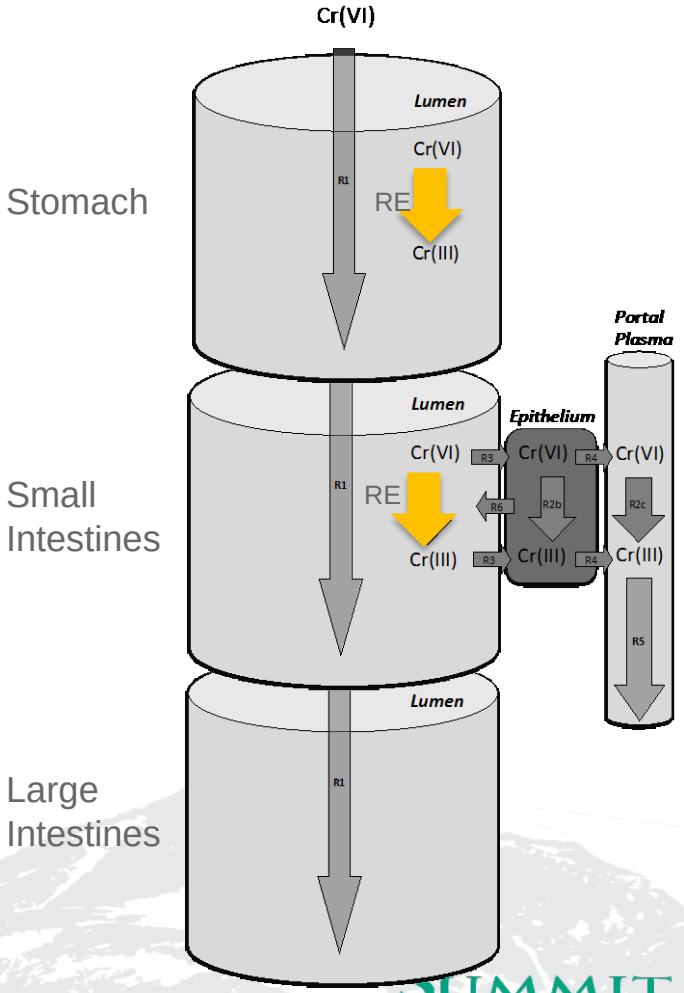
- CrVI reduction by fed human gastric fluid (GF) samples (*previous work done using fasted GF samples*)
- CrVI reduction by individual human GF samples (*previous work done using mostly pooled samples*)
- CrVI reduction by GF at elevated pH (previous work included only 1 sample above pH 4)
  - *Relationship between reduction rate constants and pH (previous work provided insufficient data at elevated pH for us to characterize completely)*
- Number of reducing agent pools present in human fed and fasted samples (*unlike our work with rodent GF, previous work with human GF assessed a narrow range of CrVI concentrations*)

CrVI reduction model is an important component of the PBPK model

$$Rate\ Reduction = C_{CrVI} x [(K_{Red} x C_{RE})_{Pool1} + \ldots (K_{Red} x C_{RE})_{PoolN}]$$

Where,

- C_{CrVI} = concentration of CrVI (mg/L)
- K_{Red} = second order rate constant for reduction (L2/mg-hr); pH-dependent;
- C_{RE} = concentration of reducing equivalents (reduction capacity, mg/L)
- N = number of pools (values for KRed and CRE differ between pools)



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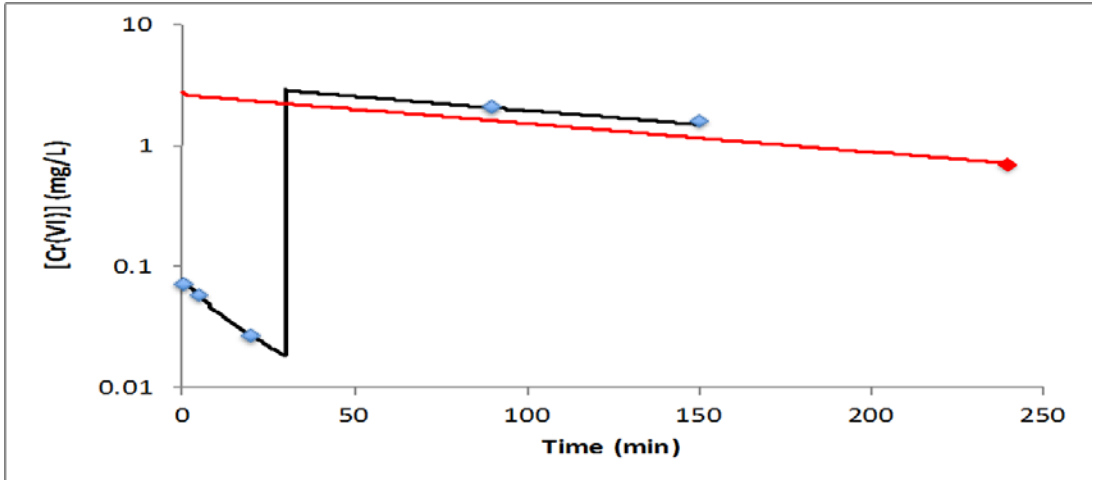
New PK Data: Ex Vivo Reduction Studies in Human Gastric Fluid Samples



Ex vivo reduction data collected using human GF obtained from Dr. Silvio DeFlora:

- *Includes fed (n=8) and fasted (n=5) individual GF samples, which fills important data gaps (sample figure shown here)*
- *Also include individual proton pump inhibitor (PPI; Prilosec®) user samples (n=3; Duke University)*
- *Assessed using dual spike design*

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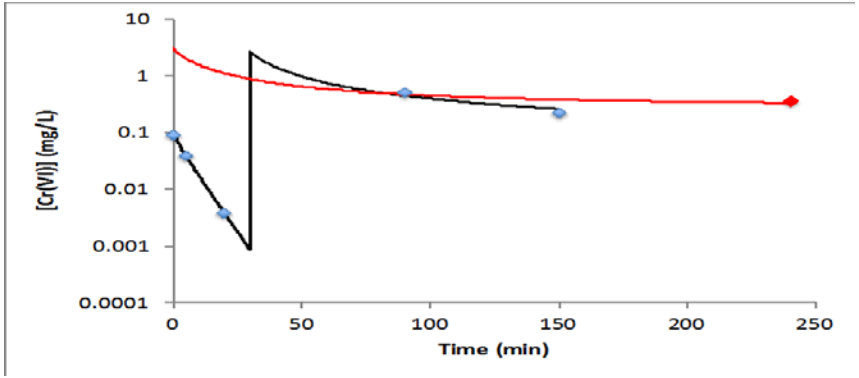
New PK Data: Ex Vivo Reduction Studies in Human Gastric Fluid Sample



The dual-spike design provides very useful data:

- In most cases, CrVI is more efficiently reduced at low concentrations than at high concentrations.
  - *Note differences in slope for CrVI reduction in the 1st 30 minutes compared to after 30 minutes)*
- Allows for characterization of rates and capacities of multiple reducing agent pools in human samples (data gap)
- Depletion of reducing agents reflects an important source of nonlinear toxicokinetics that needs to be considered when attempting to extrapolate the observations from the NTP bioassay (high concentrations) to environmental exposures (low concentrations)

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New Data: Number of Pools

Sample Group	Number of Reducing Agent Pools	Reducing Agent Pools			Maximum LL For All Samples	Number of Estimated Parameters (each sample optimized individually)	AIC
		Pool 1 (fast)	Pool 2 (slow)	Pool 3 (very slow)			
Experiment 2A (n=8 human fed samples)	1	1st order	NA	NA	-2452	8	4921
		2nd order	NA	NA	-782	16	1597
	2	2nd order	1st order	NA	-18	22	79
		2nd order	2nd order	NA	6	30	48
	3	*2nd order*	*2nd order*	*1st order*	*88*	*32*	*-112*
		2nd order	2nd order	2nd order	88	40	-95
Experiment 2B (n=3 human PPI samples)	1	1st order	NA	NA	-159	3	325
		2nd order	NA	NA	28	6	-44
	2	2nd order	1st order	NA	53	9	-89
		2nd order	2nd order	NA	75	12	-126.0
	3	*2nd order*	*2nd order*	*1st order*	*78*	*15*	*-126.3*
		2nd order	2nd order	2nd order	78	18	-120
Experiment 2C (n=5 human fasted samples)	1	1st order	NA	NA	-3659	5	7328
		2nd order	NA	NA	-54	10	127
	2	2nd order	1st order	NA	-51	15	133
		2nd order	2nd order	NA	31	20	-22
	3	*2nd order*	*2nd order*	*1st order*	*42*	*25*	*-35*
		2nd order	2nd order	2nd order	33	30	-5

- For fed, fasted, and PPI samples a 3-pool model provided the best fit (lowest AIC) to the data collected
- This is consistent with conclusions for rat and mouse GF data (Schlosser and Sasso, 2014)

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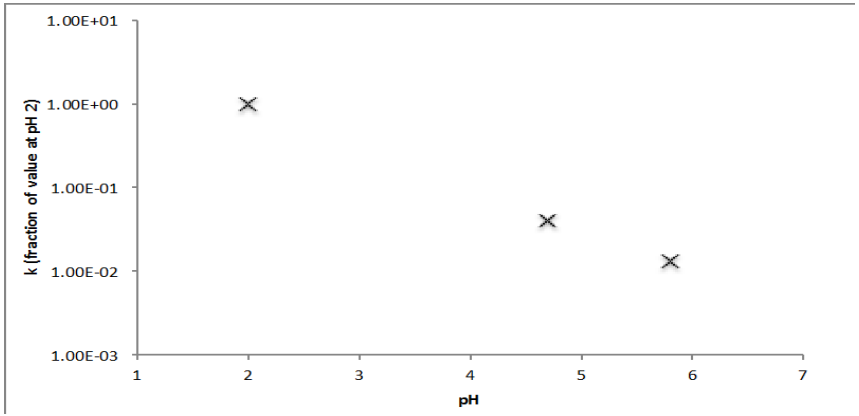
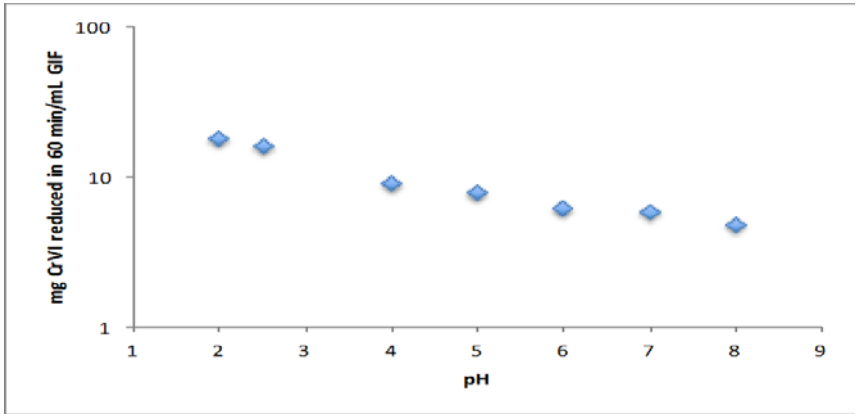
New Data: CrVI Reduction at Elevated pH

(A)



(B)



To characterize CrVI reduction at elevated pH:

- We artificially increased the pH of a sample (innate pH = 2) to 4.7 and 5.8 and estimated the change in k (Panel A)
- Independently, De Flora et al. increased the pH of a different sample (innate pH=2) to 2.5-8 and measured the amount of CrVI reduced in 60 min (proportionate to k) (Panel B)
- Ex vivo reduction runs using individual PPI samples (pH 5.8-7.5) (part of data discussed in slide #6)

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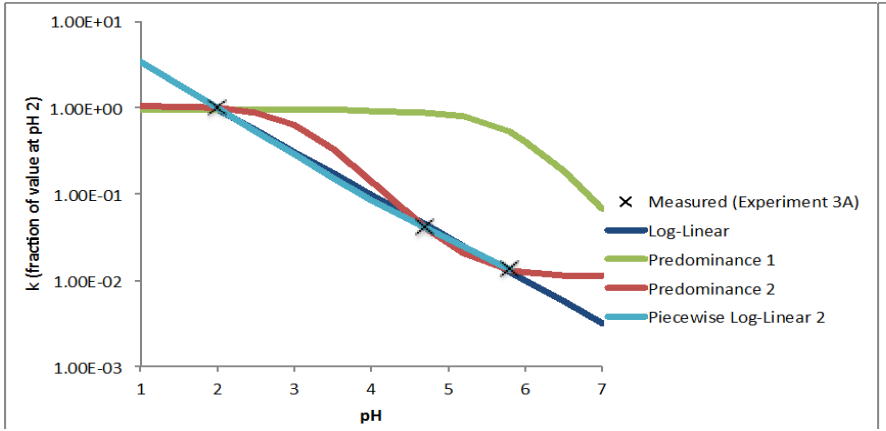
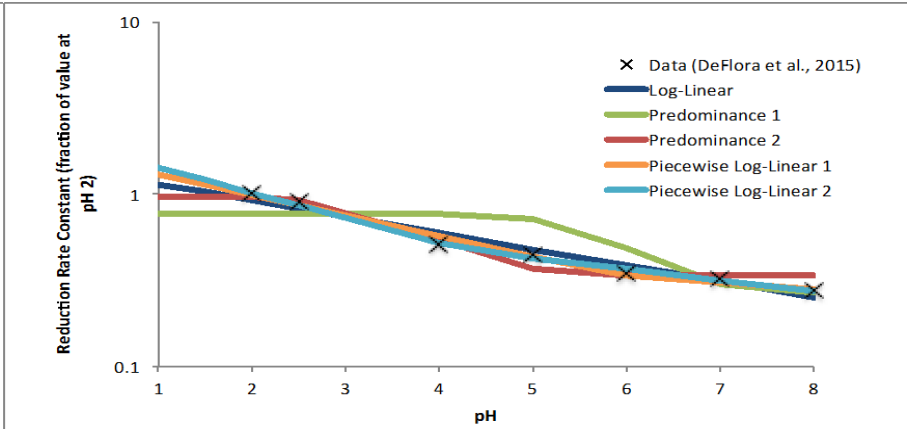
New Data: Revised pH-Dependence for the Reduction Rate Constant in Human GF

Our Data



De Flora Data



- We evaluated several pH-dependent forms against these GF data
  - Log-linear (form used in Kirman et al., 2013)
  - Predominance 1 & 2 (forms proposed by Schlosser and Sasso)
    - Based on the pH-dependent forms of chromate present
  - Piecewise log-linear (new)
    - Allows for an inflection point

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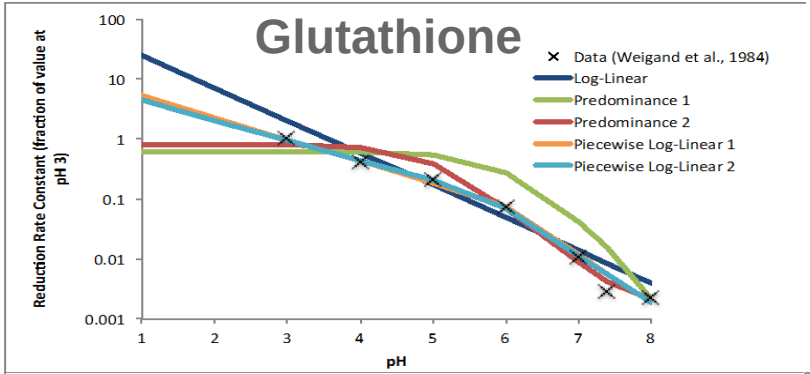
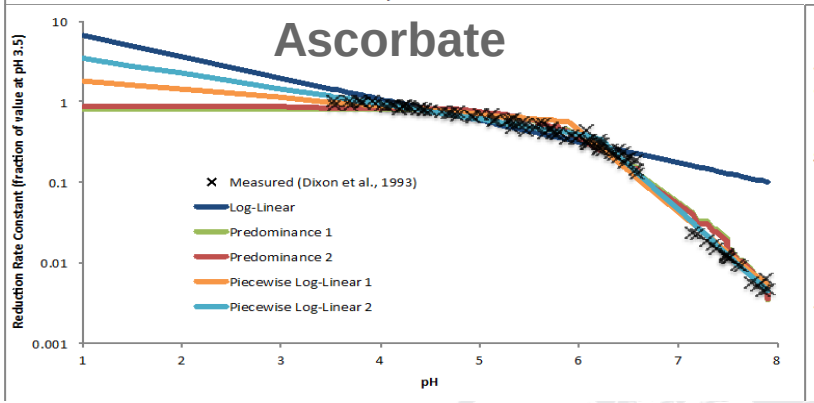
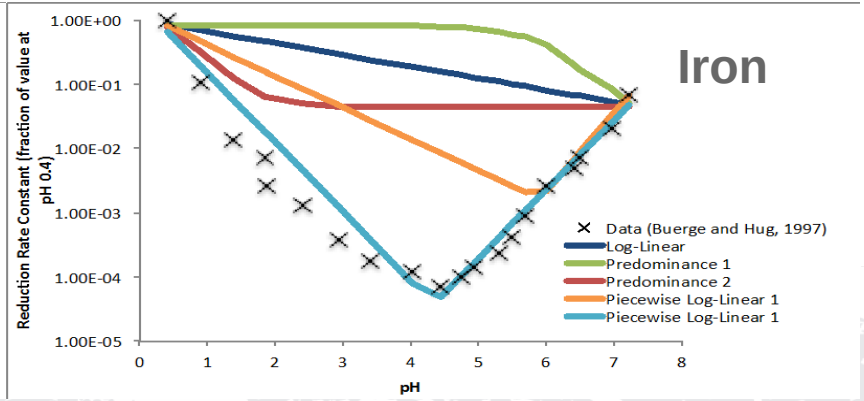
Revised pH-Dependence for the Reduction Rate Constant for Specific Reducing Agents







We also evaluated several pH-dependent forms against published data sets for specific reducing agents (glutathione, ascorbate, iron), which are likely components of human GF

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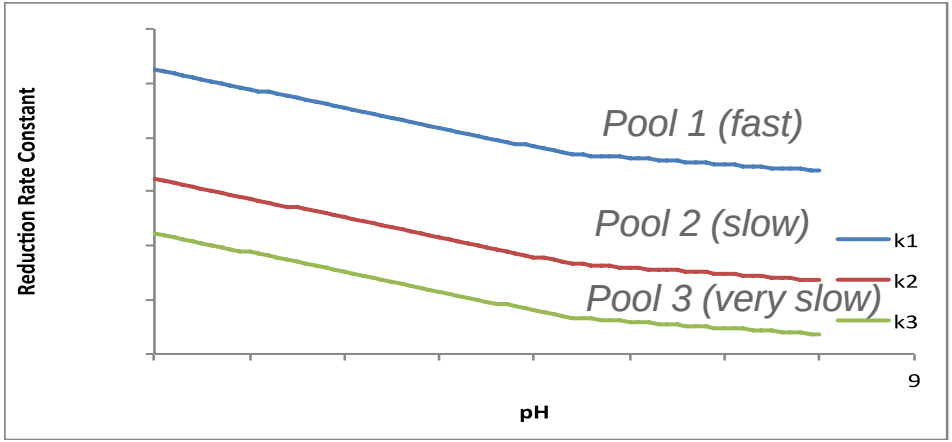
Revised pH-Dependence for the Reduction Rate Constant



Based on this evaluation we selected the piecewise log-linear model for all three reduction constants (fast, slow, and very slow pools)

- Piecewise log-linear (replaces simpler log-linear model)
- Differs from USEPA's Cr predominance model, but may not produce meaningful difference in the risk assessment

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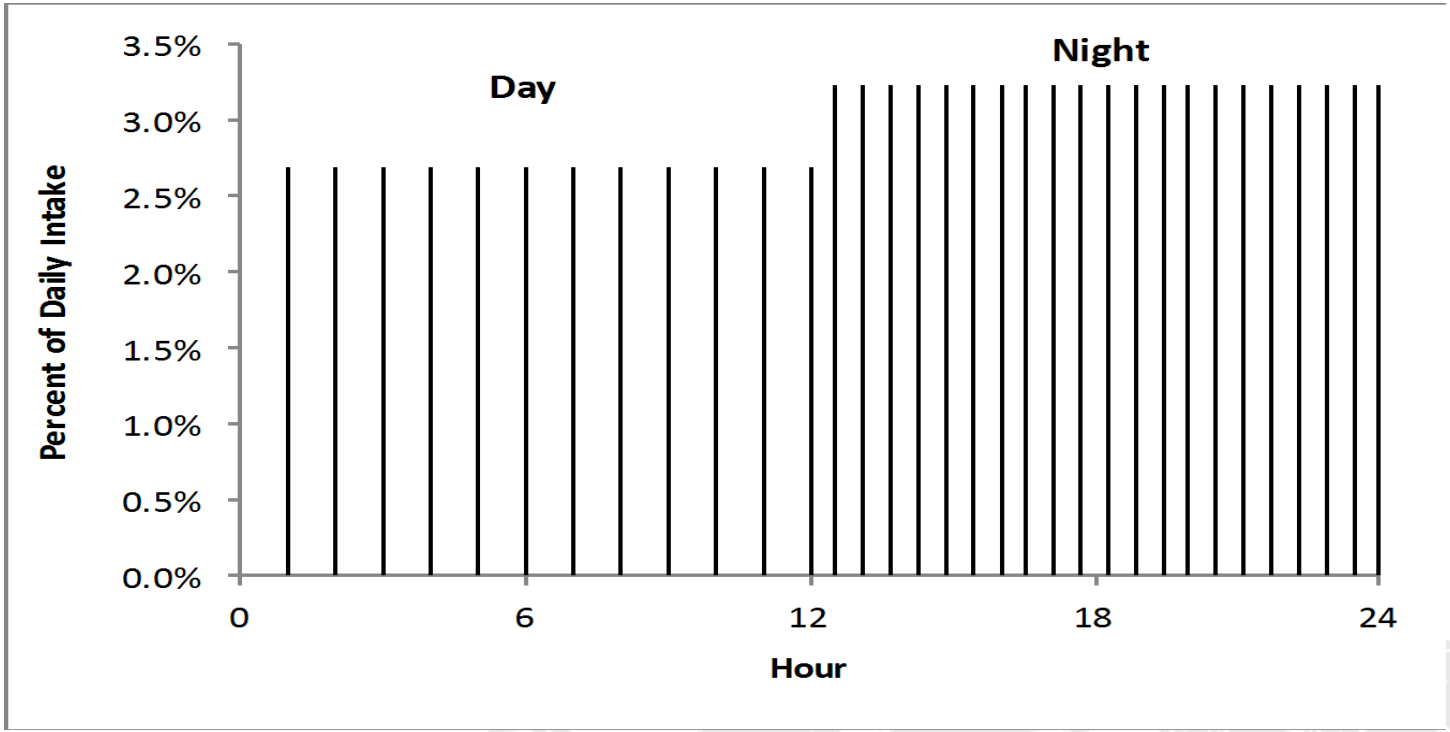
Reduction Capacities

		Reducing Equivalents Concentration (mg/L)	
Species for GF Samples	**Reducing Agent Pool (reaction rate)**	**Fed**	**Fasted**
Human	Pool 1 (fast)	0.68±0.76	2.6±2.8
	Pool 2 (slow)	27±28	12±18
	Pool 3 (very slow)	Unlimited[1]	Unlimited[1]
Mouse[2]	Pool 1 (fast)	6.1	NA
	Pool 2 (slow)	27	NA
	Pool 3 (very slow)	Unlimited[1]	NA
Rat[2]	Pool 1 (fast)	7.1	NA
	Pool 2 (slow)	73	NA
	Pool 3 (very slow)	Unlimited[1]	NA

- Based on a capacity of ~0.7 mg/L for RE pool 1 (fast reaction) in fed human GIF samples, we predict CrVI to be more efficiently reduced at low concentrations (<0.7 mg/L) than at high concentrations (>0.7 mg/L)

PBPK Refinement: Simulating Individual Drinking Water Events in Mice (Gannon et al., 1992)



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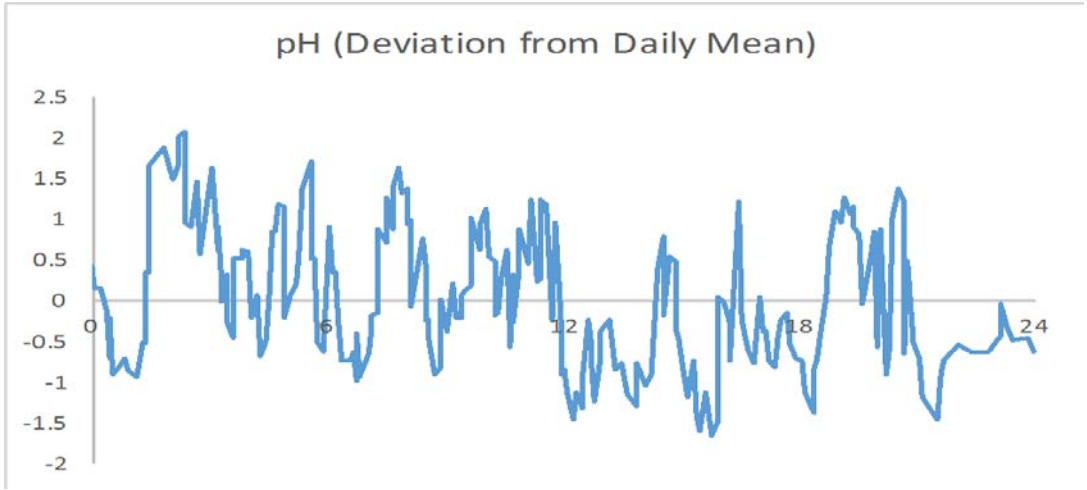
PBPK Refinement: Temporal Variation in Rodent Gastric pH



- Based on data of Rudholm et al (2008)
- Data identified to help address comment/question regard diurnal variation in rodent gastric pH
- No clear difference between pH during day vs night
- Currently evaluating how to best use these data, and whether this degree of complexity makes a meaningful difference to modeling results

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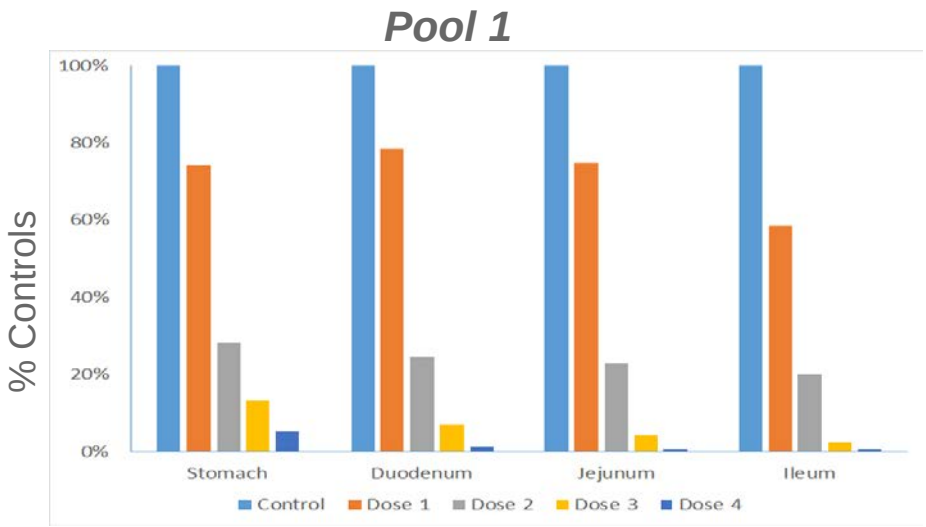
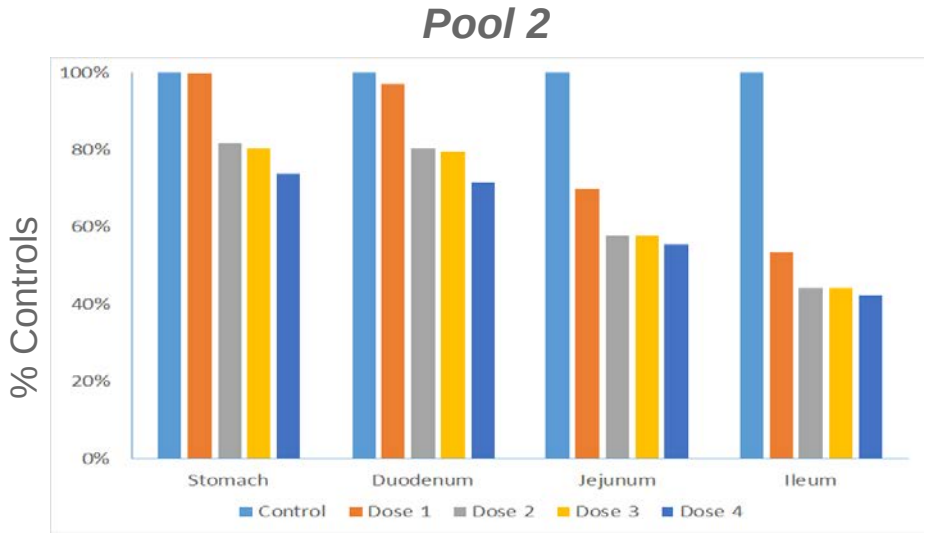
Preliminary PBPK Model Predictions: Depletion of Reducing Agent Pools





Under conditions of NTP bioassay, PBPK model predictions show:

- Significant depletion of Pool 1 (fast reaction) reducing agents
- Some depletion of Pool 2 (slow reaction) reducing agents

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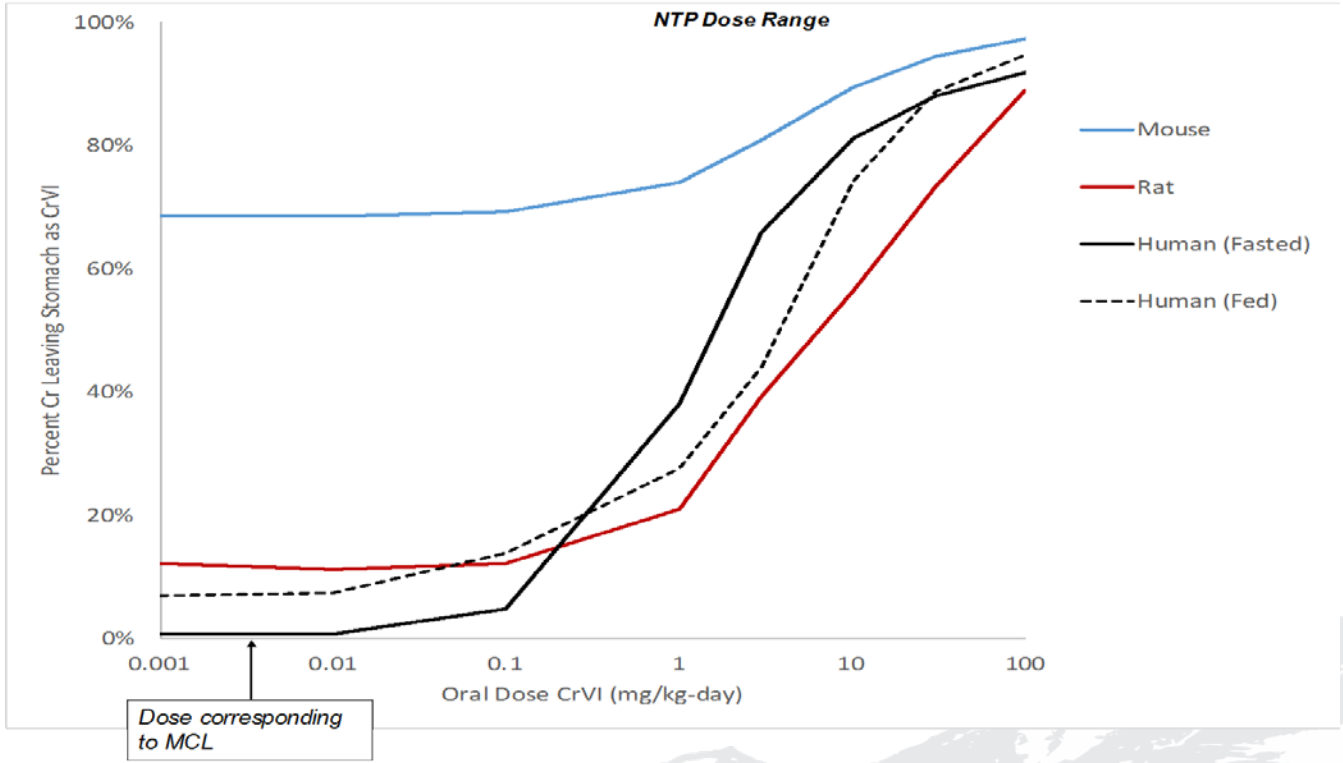
Using the PBPK Model to Characterize Species Differences and Nonlinear Toxicokinetics



- *Due to shorter GI transit times (less time for reduction to occur), a higher percentage of CrVI reaches the small intestines in mice (blue curve) compared to other species*
- *Due to reducing agent depletion, a greater percentage of CrVI reaches the small intestines in all species*
- *Nonlinear toxicokinetics become important at oral doses near 0.1 mg/kg-day (above human GF capacity of 0.7 mg/L for fast reduction reaction)*

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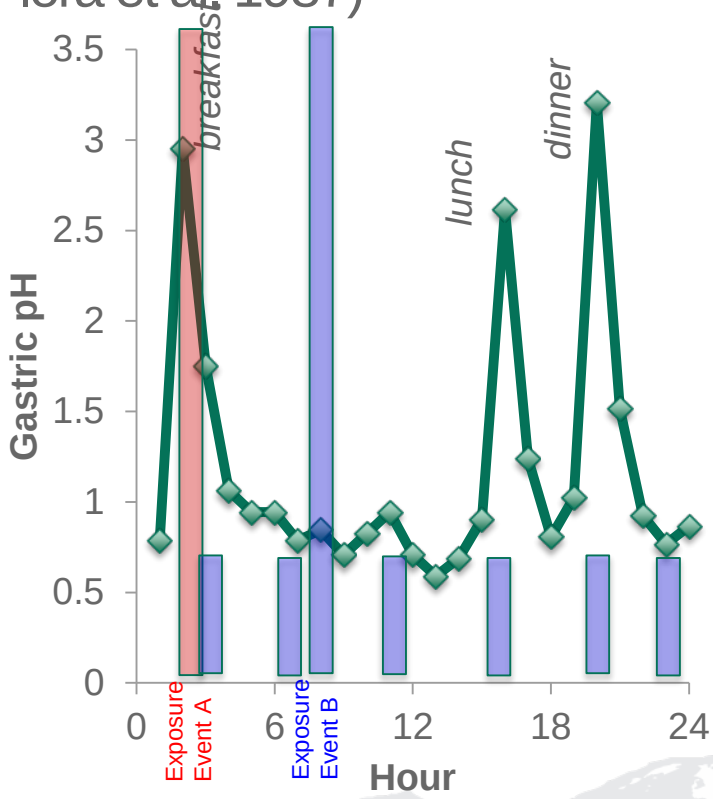
Using the PBPK Model to Address Temporal Variation, Inter-individual Variation, and Sensitive Subpopulations

- Temporal Variation
  - *Impact of meals on gastric pH*
- Inter-individual variation
  - *Age differences*
- Sensitive Subpopulations:
  - *Neonates*
  - *Proton pump inhibitor (PPI) Users*
  - *Hypochlorhydria*

Temporal/Diurnal Variation in Gastric pH (data from de Flora et al. 1987)



- *Gastric pH is important since the rate Cr(VI) reduction is pH-dependent*
- *In addition, other GI factors exhibit temporal variation*
  - ***Gastric Transit Rates:*** *Slower when food is present, faster with liquid intake only*
  - ***Reducing Agents:*** *concentrations of ascorbate/sulfhydryls depend on the presence or absence of food in GI tract*
- *Timing of exposure events affects pyloric flux (dose delivered to SI)*

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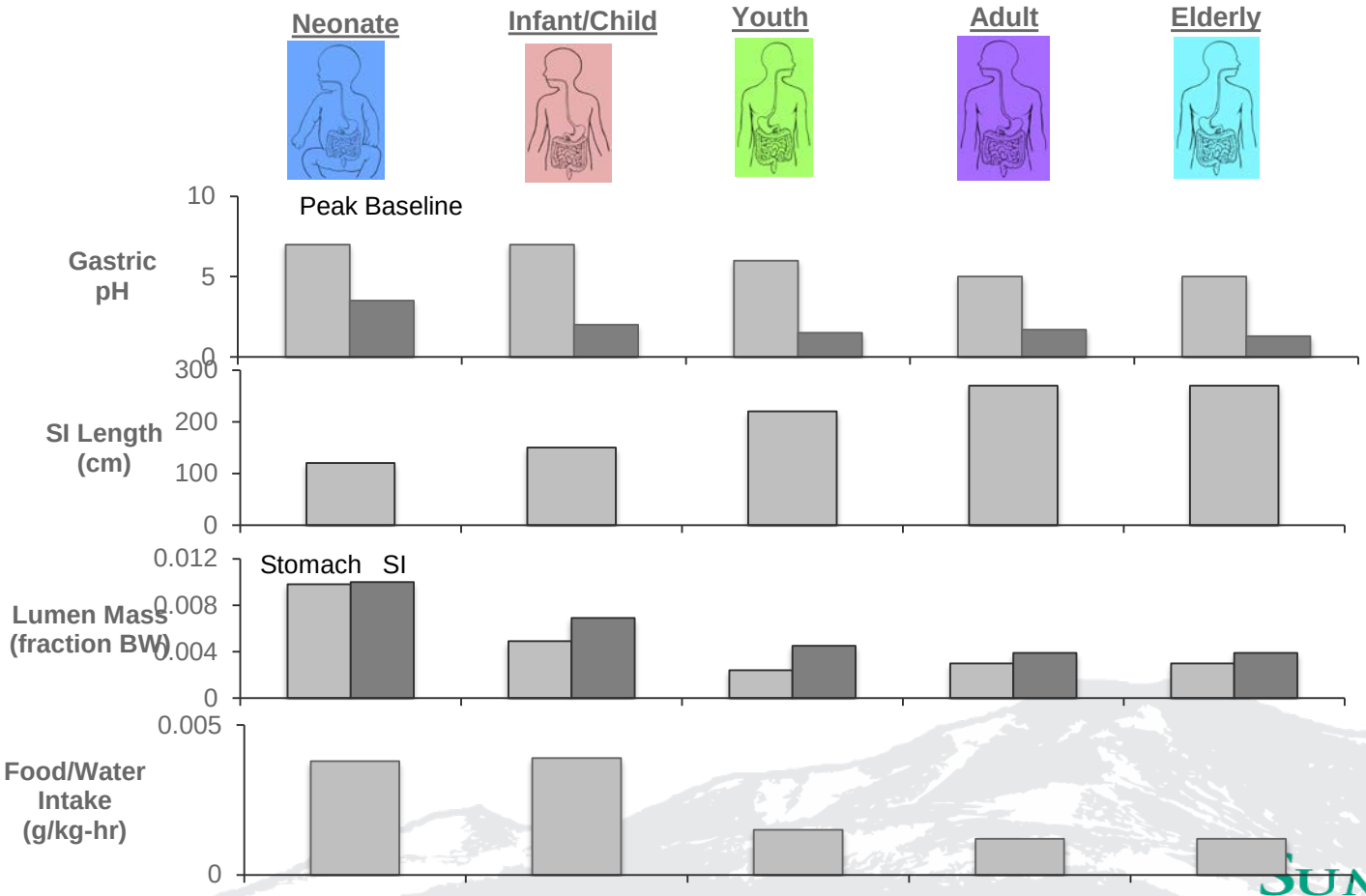
Variation in Model Parameters Across Lifestage



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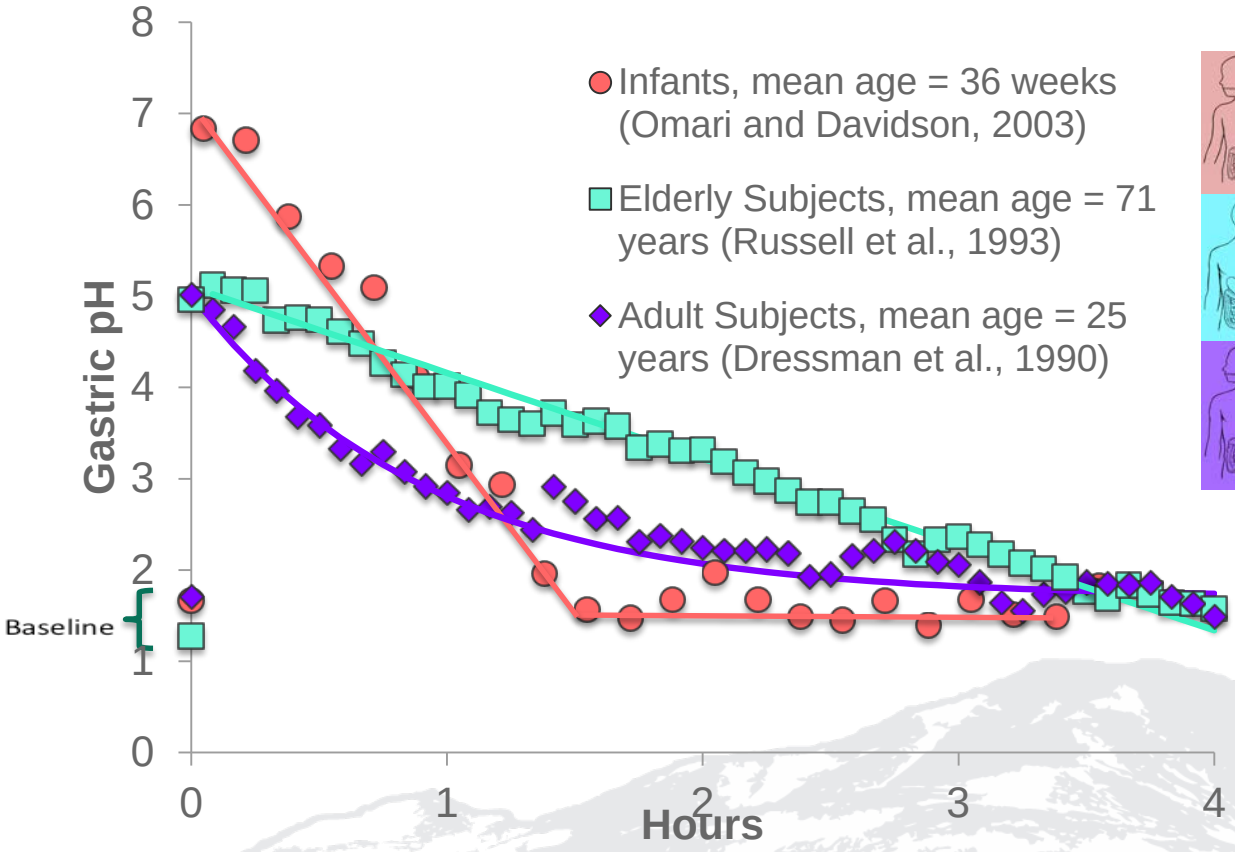
Variation Across Lifestage: Gastric pH After a Meal



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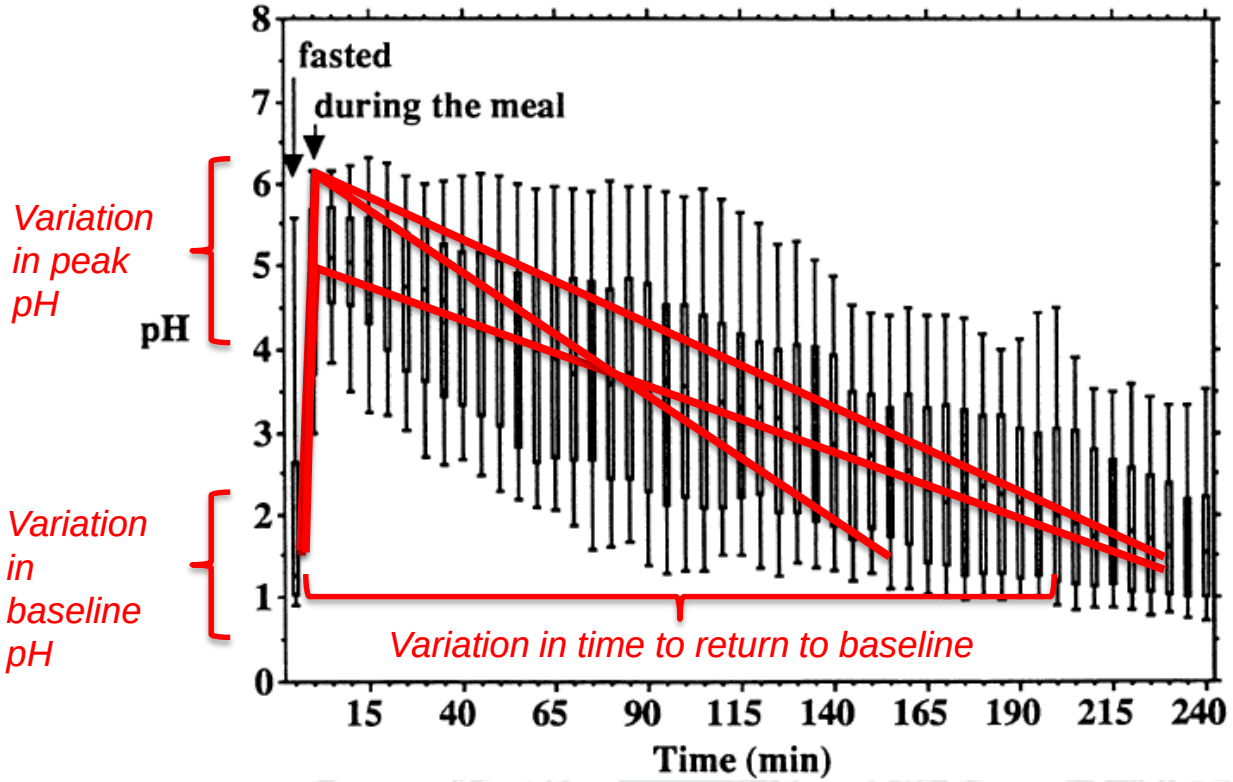
Individual Variation: Gastric pH After a Meal (Dressman et al., 1990; Russell et al., 1993)



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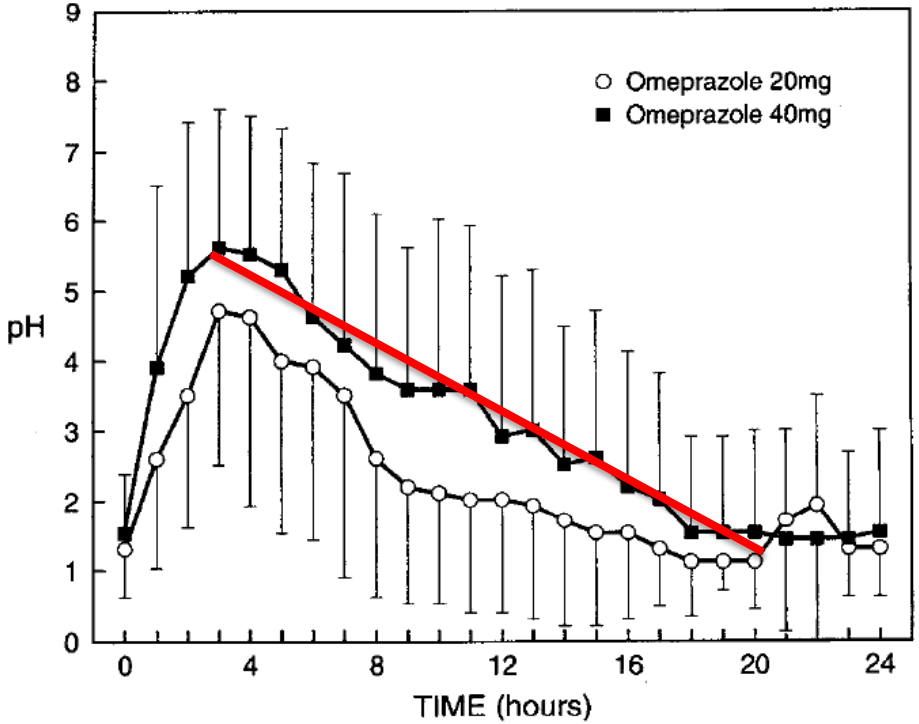
Sensitive Subpopulations: Proton Pump Inhibitor (PPI) Users (Atanassoff et al. 1995)



PPI user compared to untreated adults:

- *Higher peak pH*
- *Greater variation in peak pH*
- *<u>Much</u> slower return to baseline (16 hours vs 3 hours)*

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Hypochlorhydria

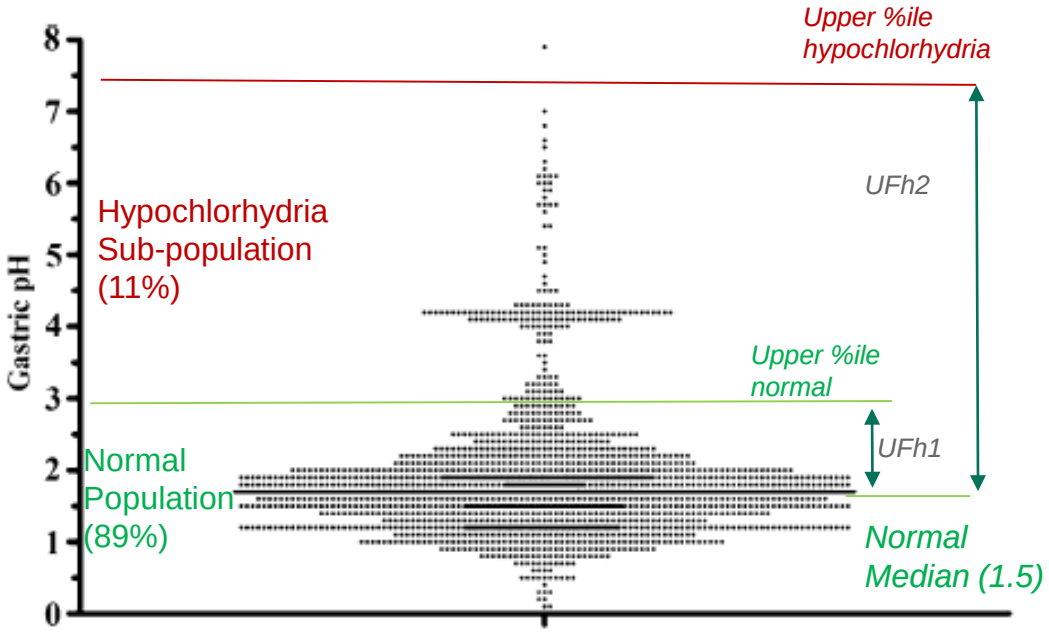

Fig. 2 Gastric pH scatter plot for 1,582 symptomatic subjects showing a bimodal distribution

- Data in Fig 2 are from Ayazi et al 2009
- Bimodal distribution; authors identify pH=2.9 as cutoff (95%ile)
- Hypochlorhydria is a chronic condition whose prevalence increases with age: 0-4 yrs (~1%); 5-9 yrs (~6%); 10-15 yrs (~8%) (Seo et al 2015); Adults (~11%; Ayazi).
  - Higher prevalence w/ age dependence in Japanese (Morihara et al. 2001)
  - In some individuals elevated pH may be intermittent (Hurwitz et al., 1997)
- In Thompson et al. (2014), we used a default value of 10 for UFh (3 each for TK & TD). We intend to replace the TK component with a data-derived UF values
  - Use pH variation to estimate variation in dose measures
  - Model hypochlorhydria as a separate population
  - Calculate two values for UFh for each dose measure (as depicted in figure).

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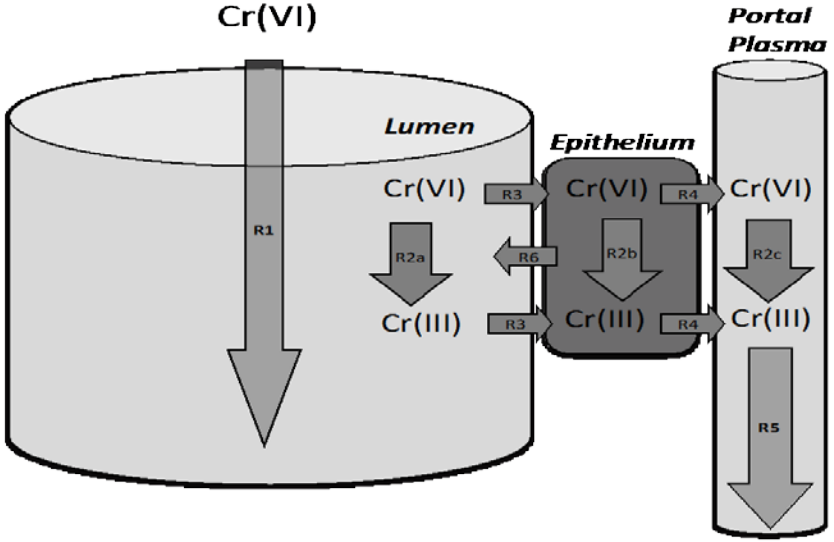
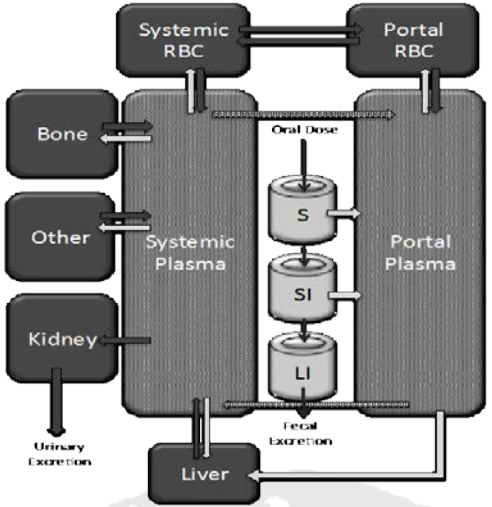
2016 PK Publication Plans

- Ex Vivo Reduction Studies: Companion papers published in summer 2016
  - *Kirman et al. (2016): ex vivo reduction SIDMS data and modeling*
  - *De Flora et al. (2016): ex vivo reduction colorimetric data*
- Updated PBPK Model:
  - *In prep, submit 2016*
  - *Will supersede Kirman et al. (2012, 2013)*
- PBPK Application/Risk Assessment:
  - *In prep, submit 2016*
  - *Will supersede Thompson et al. (2014)*

Questions on PK?





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Genotoxicity

IWGT Recommendations for *In Vivo* Genotoxicity Assays

Mutation Research 783 (2015) 66–78

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

IWGT report on quantitative approaches to genotoxicity risk assessment II. Use of point-of-departure (PoD) metrics in defining acceptable exposure limits and assessing human risk☆

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ABSTRACT

This is the second of two reports from the International Workshops on Genotoxicity Testing (IWGT) Working Group on Quantitative Approaches to Genetic Toxicology Risk Assessment (the QWG). The first report summarized the discussions and recommendations of the QWG related to the need for quantitative dose–response analysis of genetic toxicology data, the existence and appropriate evaluation of threshold responses, and methods to analyze exposure-response relationships and derive points of departure (PoDs) from which acceptable exposure levels could be determined. This report summarizes the QWG discussions and recommendations regarding appropriate approaches to evaluate exposure-related risks of genotoxic damage, including extrapolation below identified PoDs and across test systems and species. Recommendations include the selection of appropriate genetic endpoints and target tissues, uncertainty factors and extrapolation methods to be considered, the importance and use of information on mode of action, toxicokinetics, metabolism, and exposure biomarkers when using quantitative exposure-response data to determine acceptable exposure levels in human populations or to assess the risk associated with known or anticipated exposures. The empirical relationship between genetic damage (mutation and chromosomal aberration) and cancer in animal models was also examined. It was concluded that there is a general correlation between cancer induction and mutagenic and/or clastogenic damage for agents thought to act via a genotoxic mechanism, but that the correlation is limited due to an inadequate number of cases in which mutation and cancer can be compared at a sufficient number of doses in the same target tissues of the same species and strain exposed under directly comparable routes and experimental protocols.

☆ The opinions and recommendations expressed in this publication are those of the authors, and do not necessarily reflect those of the institutions with which they may be affiliated.

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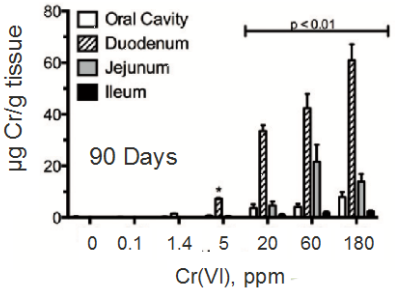
1383-5718/

- Ideally conducted in a proliferative tissue
  - Bone marrow (hematopoietic)
  - Colon
  - Stomach
  - **Small intestine (duodenum)**
- Ideally at site of carcinogenic action
  - **GI tract for Cr(VI)**
- Ideally in tissue with high dosimetry (e.g. site of contact)
  - Stomach
  - Liver
  - **Duodenum for Cr(VI)**



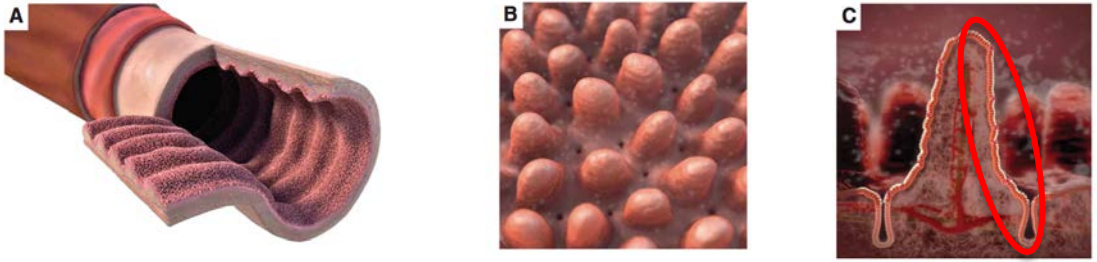
In Vivo Blood and Bone Marrow Micronucleus (MN) Data for Cr(VI)

NTP (2007) 90-day GLP Studies
B6C3F1, ≤88 ppm dw, M, (-)
B6C3F1, ≤350 ppm dw, M (-)
B6C3F1, ≤350 ppm dw, F (-)
BALB/c, ≤88 ppm dw, M, (-)
Am3-C57BL/6, ≤88 ppm dw, M, (+)

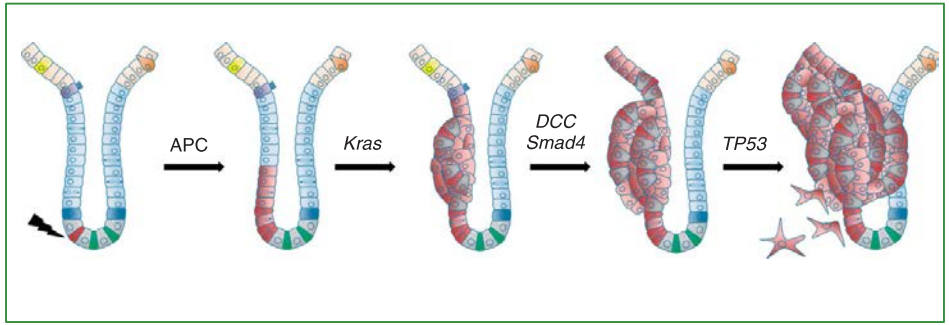
dw, drinking water

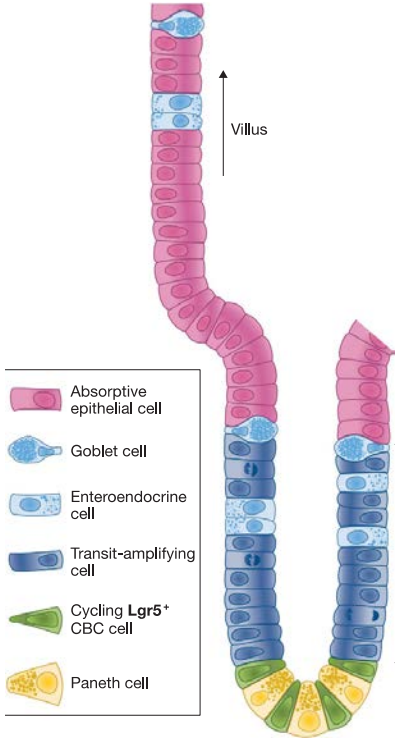
De Flora et al. (2006) Mut Res
BDF1, 20 ppm dw, 20 days, M, (-)
BDF1, 500 ppm dw, 7 mo, M, (-)
BDF1, 500 ppm dw, 7 mo, F, (-)
BDF1, 50 mg/kg gavage, 24 hr, M, (-)
Swiss albino (preg), 20 ppm dw, 17 days, (-) • Fetal PCE (liver, blood), (-)
BDF1, 50 mg/kg i.p., 24 hr, M, (+)
Swiss albino (preg), 50 mg/kg i.p., 24 hr, (+) • Fetal PCE (liver, blood), (+)

Small Intestine Structure, Biology, & Carcinogenesis



Model of Intestinal Cancer Initiation & Progression

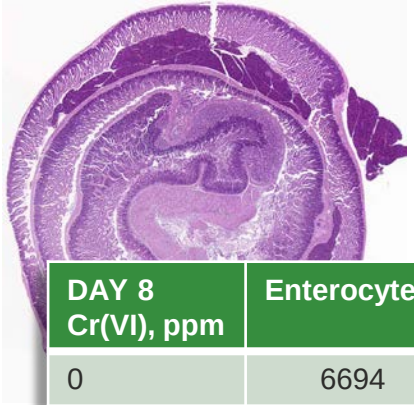




Sources: Schuijers & Clevers (2012) EMBO J. 31, 2685.
Rizk & Barker (2012) WIREs Syst Biol Med. 4, 475.

In Vivo Duodenal Micronucleus Assay (7-day Studies)

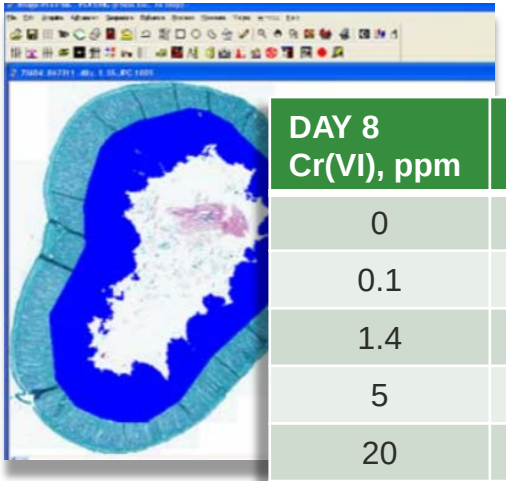
MN Study (Swiss Roll)



DAY 8 Cr(VI), ppm	Enterocytes	Crypts	Cells/Crypt	MN	KN
0	6694	171	39.3	4	0
1.4	3159	77	41.0	1	0
21	3946	76	**51.9**	1	1
180	5161	77	**67.1**	0	0
Cyclophos.	3447	87	39.3	**30**	**5**

Thompson et al. (2015) Mut Res 789-790, 61-66.

MOA Study (Transverse)



DAY 8 Cr(VI), ppm	Crypt MN, KN	Villi MN, KN
0	1, 0	1, 0
0.1	0, 0	3, 0
1.4	0, 0	5, 0
5	0, 0	2, 0
20	0, 0	1, 2
60	0, 0	6, **3**
180	0, 1	**11**, **9**

O'Brien et al. (2013) Mut Res

Note: bolded values are statistically significant

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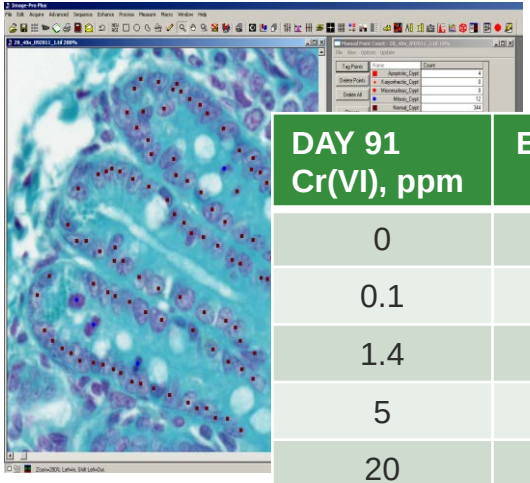
In Vivo Duodenal Micronucleus Assay (90-day Study)

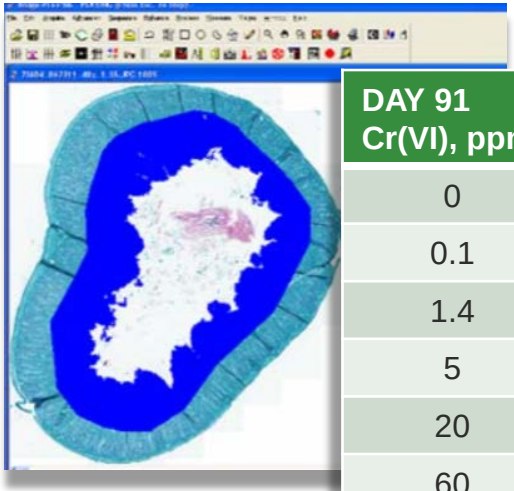
Intact Crypts



DAY 91 Cr(VI), ppm	Enterocytes	MN, KN
0	1921	0, 0
0.1	1707	0, 4*
1.4	1825	0, 0
5	1420	0, 0
20	2386	0, 0
60	2746	0, 0
180	3194	0, 0
O'Brien et al. (2013) Mut Res		

*3 observed in one animal

Full Sections



DAY 91 Cr(VI), ppm	Crypts MN, KN	Villi MN, KN
0	2, 0	1, 0
0.1	2, 1	1, 1
1.4	1, 0	2, 0
5	1, 0	0, 0
20	0, 1	2, **5**
60	0, 1	**9, 6**
180	0, 0	**9, 25**
O'Brien et al. (2013) Mut Res		

Note: bolded values are statistically significant

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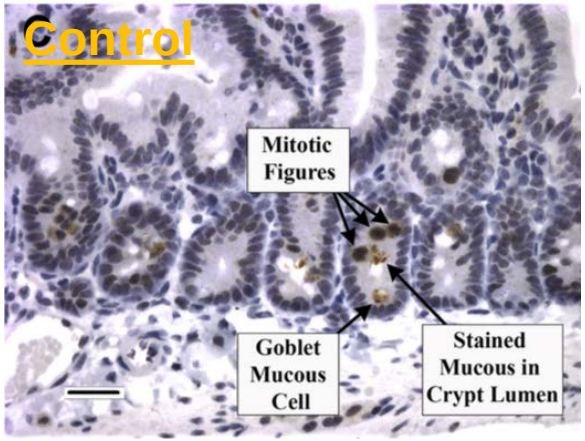
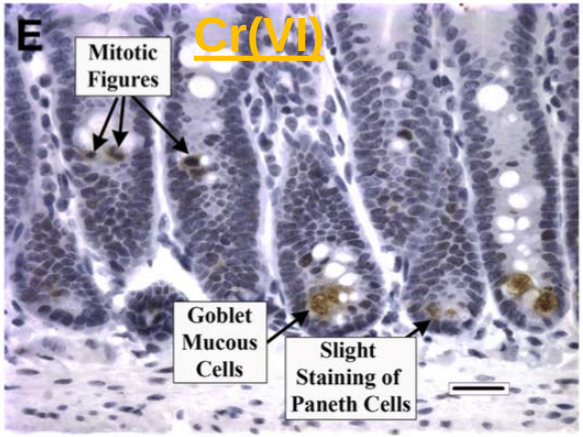
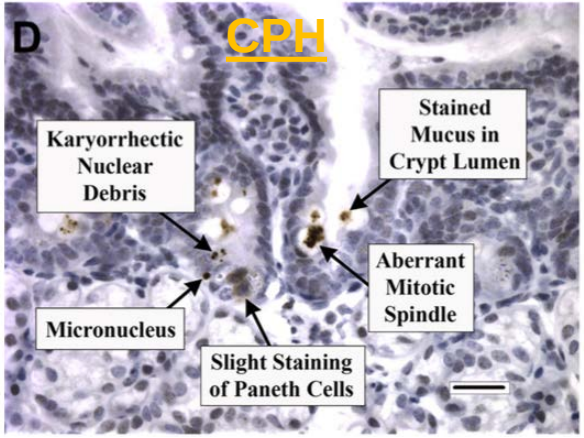
γ-H2AX Immunostaining in 7-day MN Study (Swiss Roll Sections)







γ-H2AX staining provides a ‘second look’ for aberrant nuclei.

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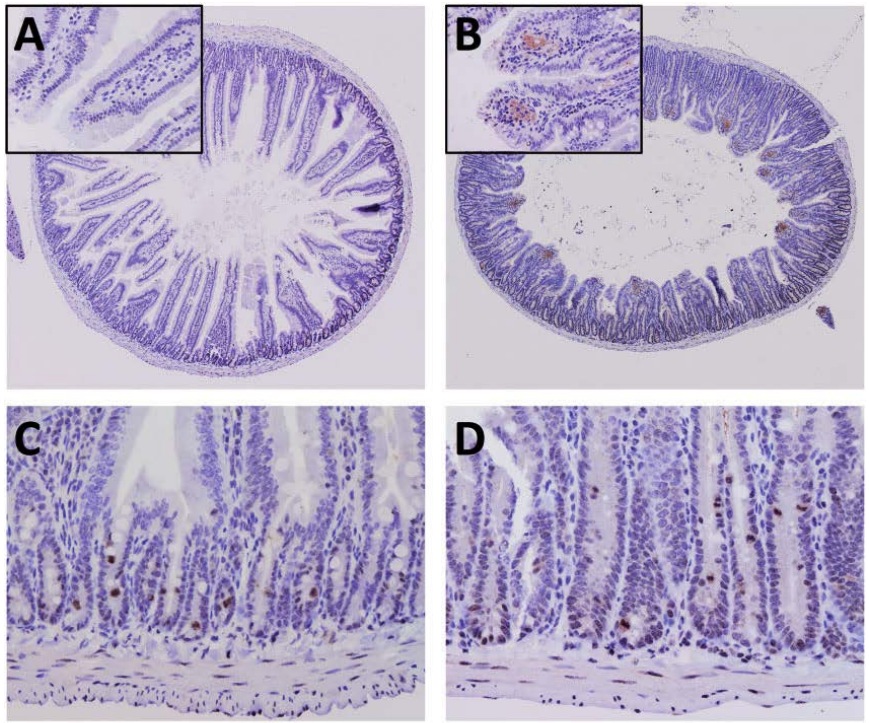
γ-H2AX Immunostaining in 90-day Study

Control

180 ppm



γ-H2AX reactivity present in villi of treated mice

γ-H2AX reactivity similar in crypts of all mice

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In Vivo K-ras Codon 12 Mutations (90-day Exposure)

No mutation data from NTP tumor tissue

K-ras selected b/c implicated in intestinal carcinogenesis

Mutations often occur in codon 12

- GGT→ GAT: spontaneous mutation; sometimes elevated with other K-ras mutations
- K-ras^{G12D} can increase proliferation in mouse intestine

Sensitive ACB-PCR assay

- B6C3F1 mice exposed to Cr(VI) for 90 days
- Codon 12 GAT mutations measured in scraped duodenal mucosa

In Vivo K-ras Codon 12 Mutations (90-day Exposure)

No mutation data from NTP tumor tissue

K-ras selected b/c implicated in

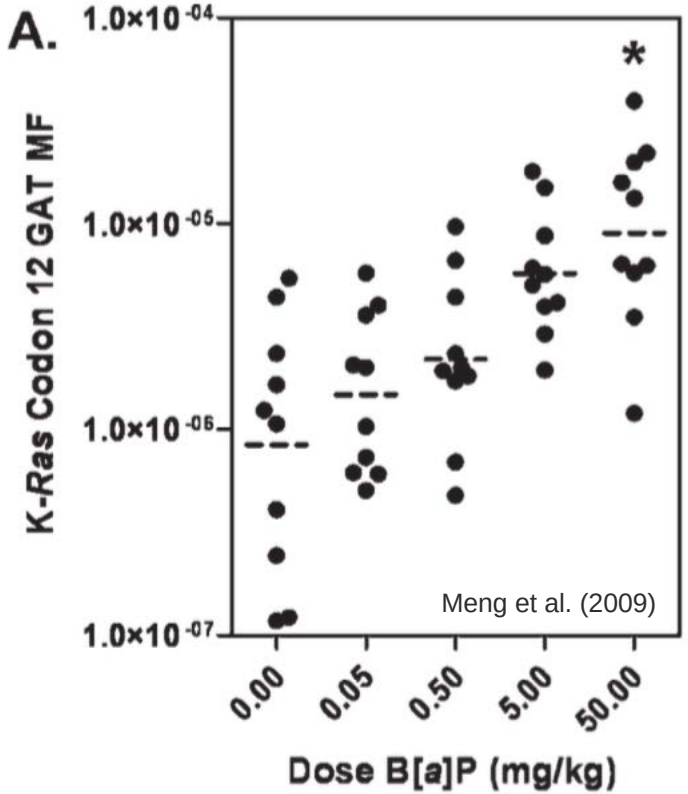
Example with B[a]P. Note trend and sig. increase in MF at highest dose.

mes

- K-ras^{G12D} can increase proliferation in mouse intestine

Sensitive ACB-PCR assay

- B6C3F1 mice exposed to Cr(VI) for 90 days
- Codon 12 GAT mutations measured in scraped duodenal mucosa



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In Vivo K-ras Codon 12 Mutations (90-day Exposure)

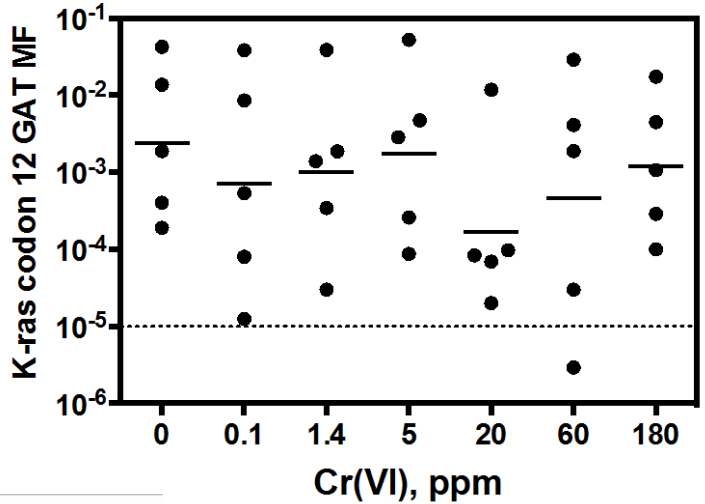
No m… tissue…

K-ras … intest…

Mutat…

- GGT→ GAT: spontaneous mutation; sometimes elevated with other K-ras mutations
- K-ras^{G12D} can increase proliferation in mouse intestine

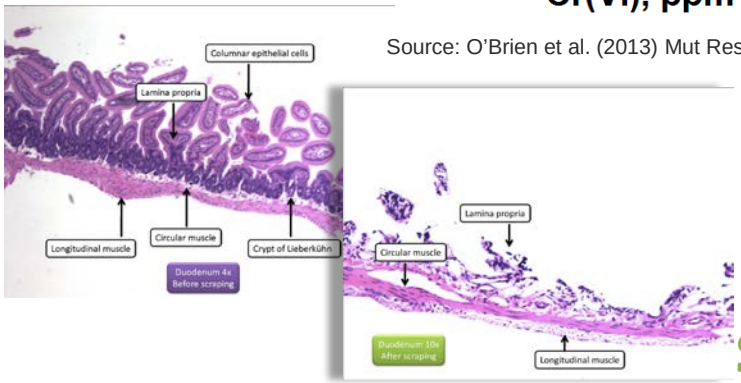
Despite high MF, small intestine tumors rare among NTP studies.



Source: O'Brien et al. (2013) Mut Res. 754, 15.

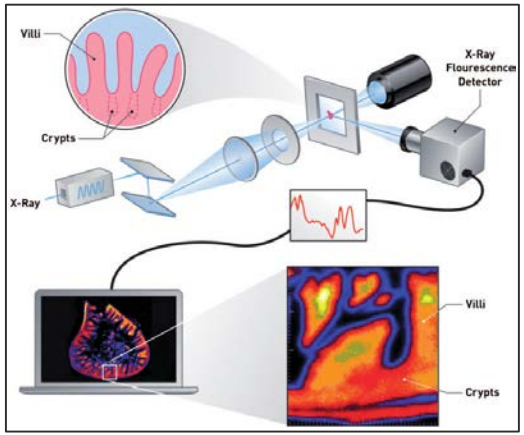
Sensitive ACB-PCR assay

- B6C3F1 mice exposed to Cr(VI) for 90 days
- Codon 12 GAT mutations measured in scraped duodenal mucosa

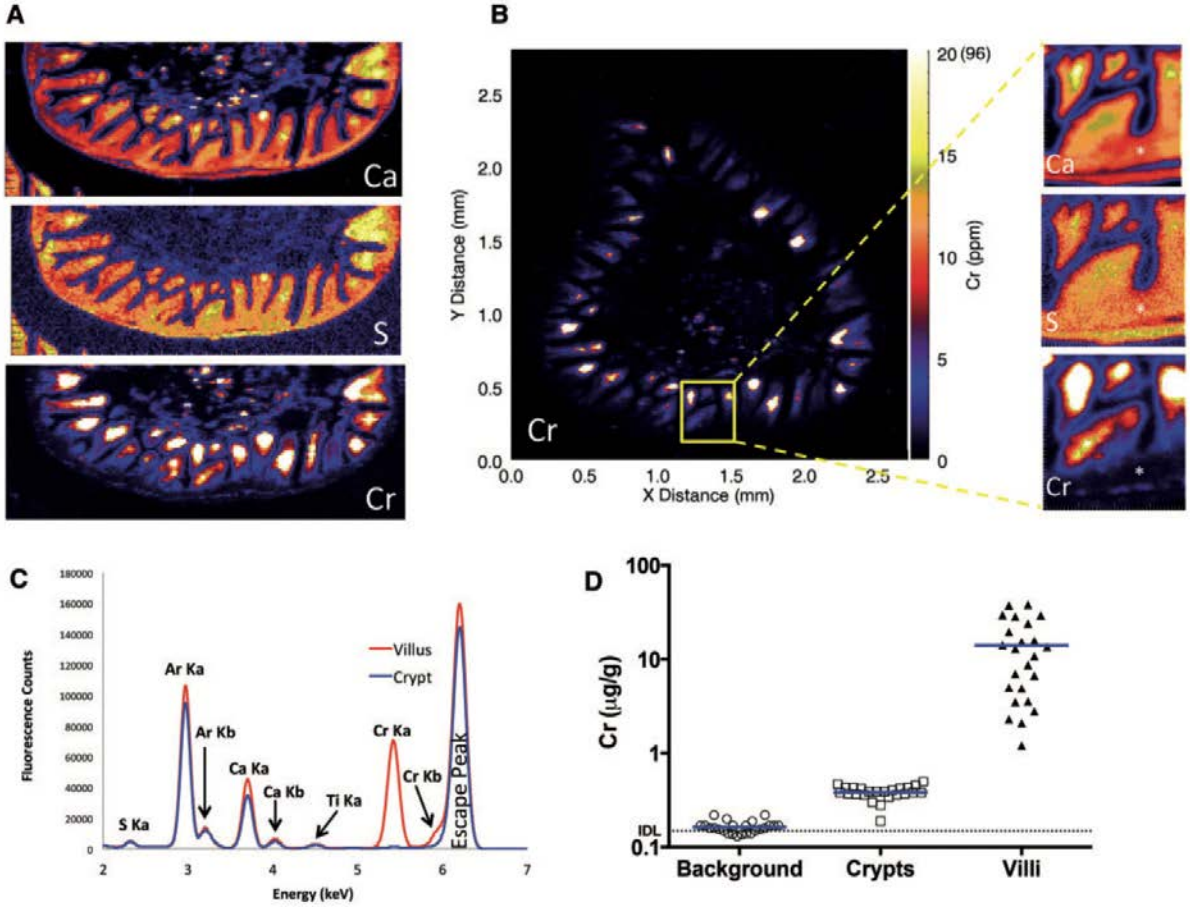


Strategies

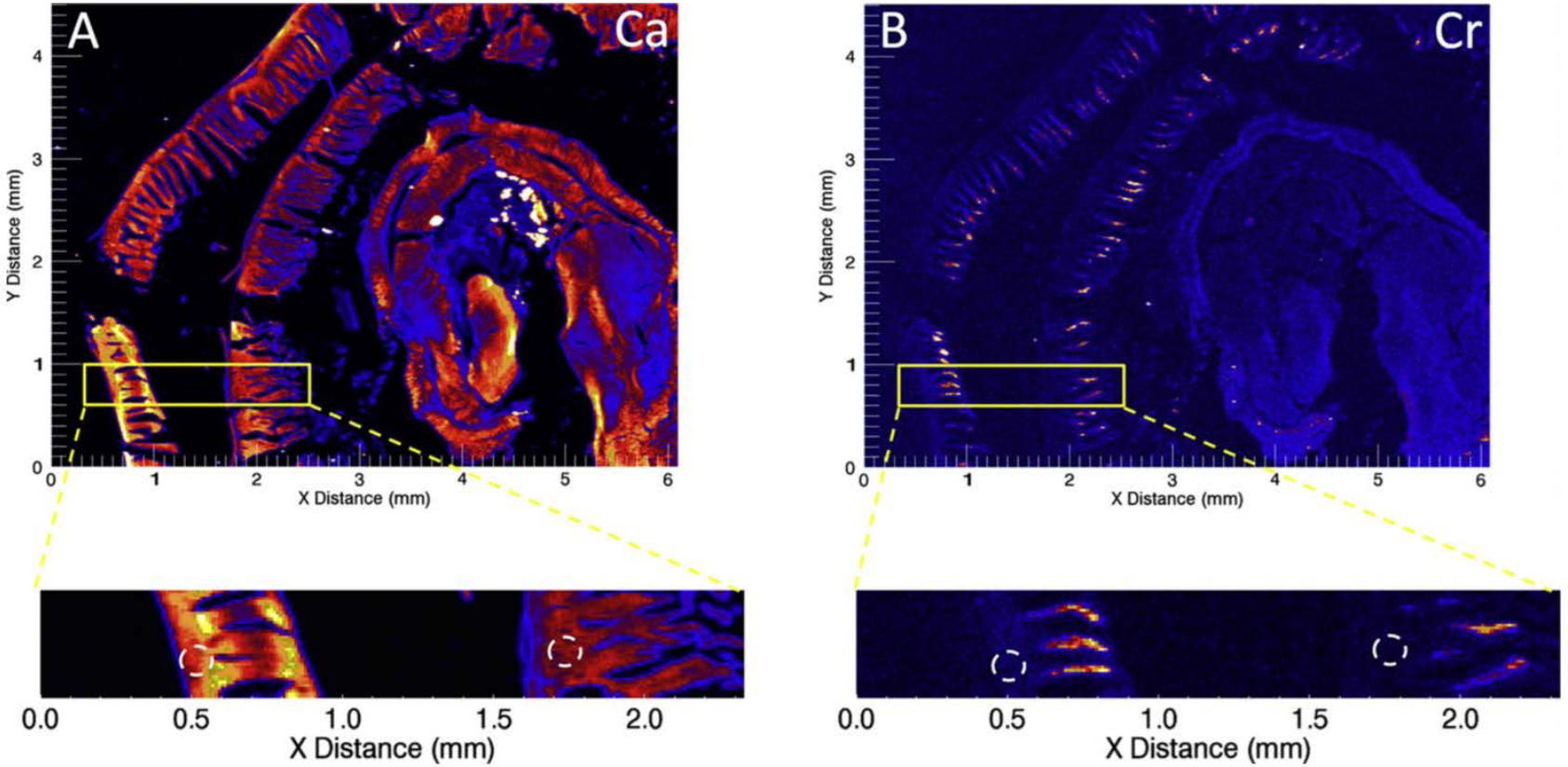
Cr(VI) Does not Reach Proliferative Crypt Compartment



Source:
Thompson et al. (2015) Tox Sci 143, 16.



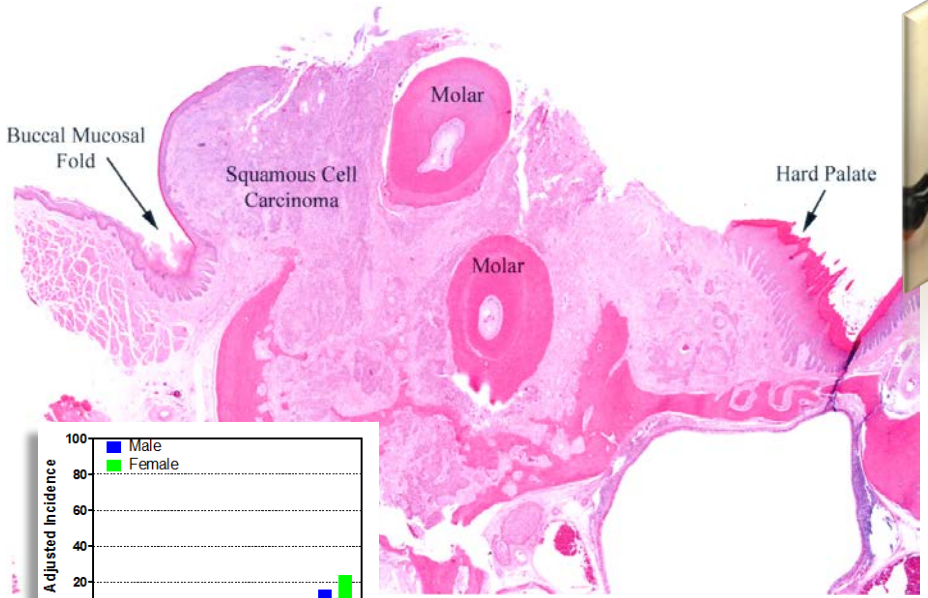
Cr(VI) Does not Reach Proliferative Crypt Compartment (7-day MN Study)



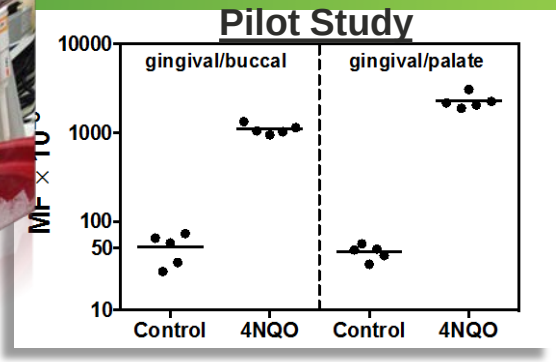
Source: Thompson et al. (2015) Mut Res 789-790, 61.

TGR Mutation Assay in Big Blue® TgF344 Rats

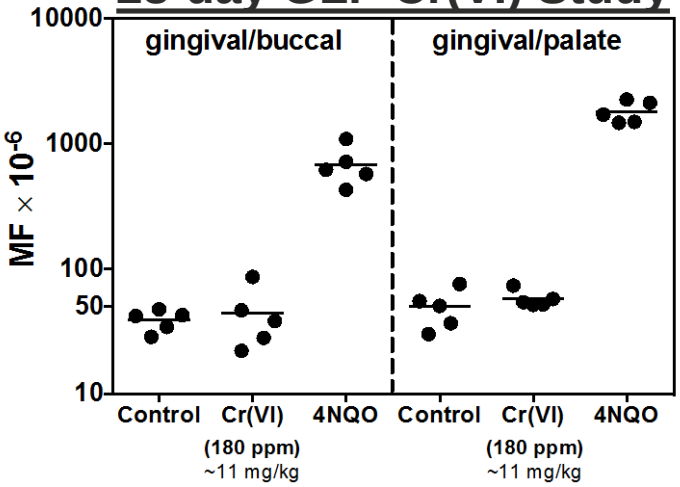
Oral Tumor Location in F344 Rats



Pilot Study

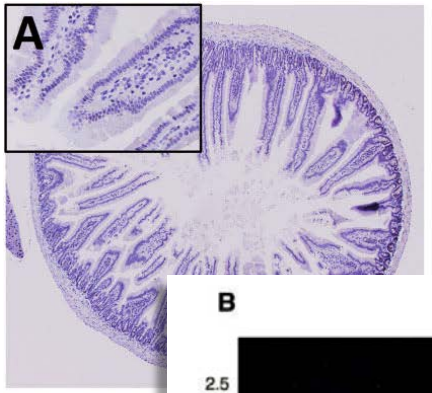


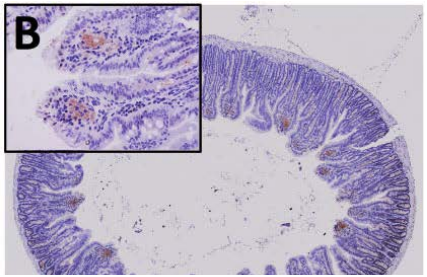
28-day GLP Cr(VI) Study

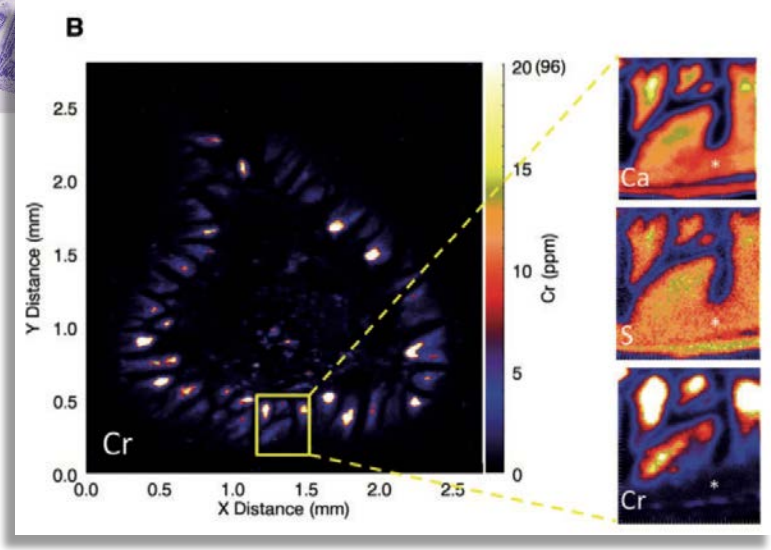


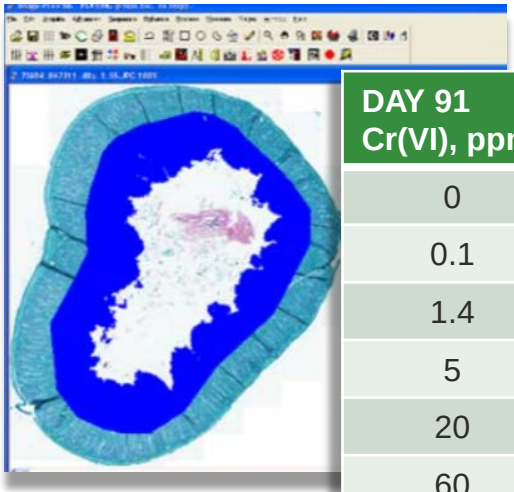
Source: Thompson et al. (2015) EMM 56, 621.

Lesions Occur in Villus at High Doses









DAY 91 Cr(VI), ppm	Crypts MN, KN	Villi MN, KN
0	2, 0	1, 0
0.1	2, 1	1, 1
1.4	1, 0	2, 0
5	1, 0	0, 0
20	0, 1	2, **5**
60	0, 1	**9, 6**
180	0, 0	**9, 25**

O'Brien et al. (2013) Mut Res

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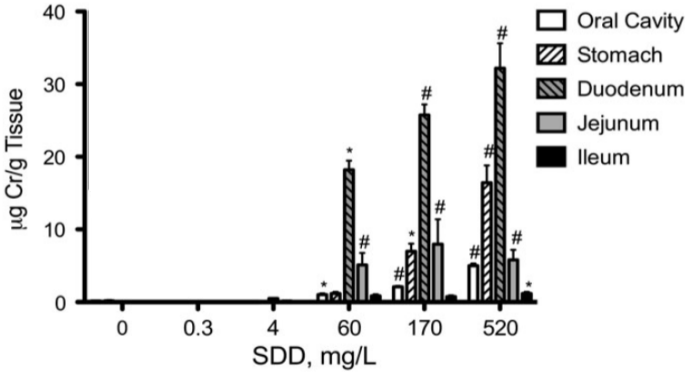
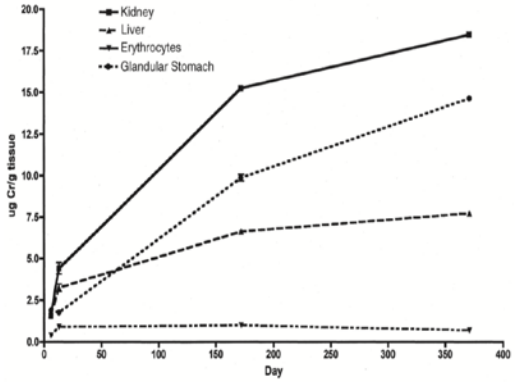
Cr6 is Also Present in Rat Villi

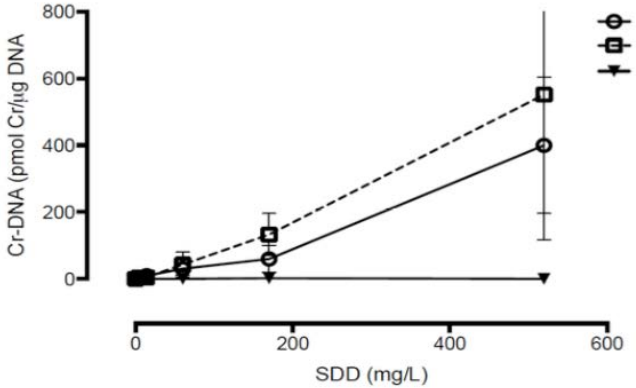
- MOA study data indicate that, like mice, highest Cr levels occur in duodenum
- MOA study data indicate that, like mice, Cr is localized to villi



- NTP 2-yr data indicate that, like mice, rats absorbed Cr (presumably from small intestine)
- NTP 2-yr rats, unlike mice, did not develop duodenal tumors
- Cr in villi (alone) does not appear sufficient to increase tumor risk in rats (same is likely true for mice)

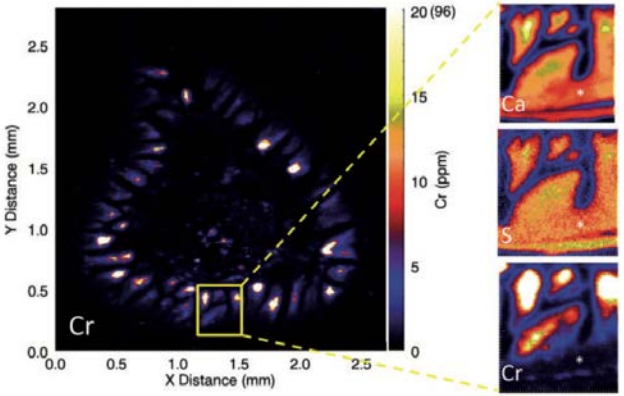


What About Cr-DNA Binding Data?



Source: O'Brien et al. (2013) Mut Res

Likely from villus cells



Issues with variability, reproducibility, and *ex vivo* Cr-DNA binding

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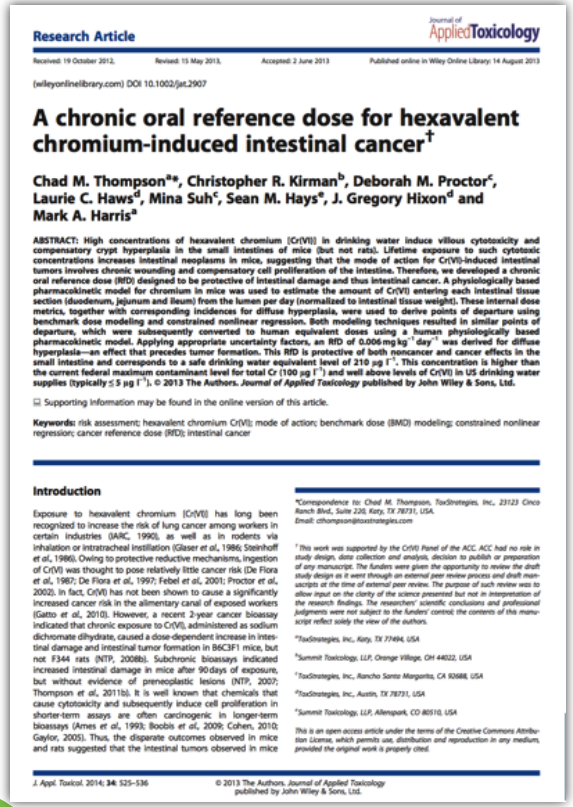
Genotoxicity Summary

- No increase in blood MN via d.w. (with exception of *Am3* strain)
- No increases in duodenal crypt MN or KN after 7 or 90 days of exposure
- No alteration of γ-H2AX crypt staining after 7 or 90 days of exposure
- No increase in k-ras codon 12 GAT MF after 90 days of exposure (crypt and villus)
- Little or no Cr is detected in intestinal crypts
- No evidence of genotoxicity in crypt
- No increase in MF in Big Blue rats after 28 days of exposure
- Increase in villus MN and KN at higher exposure doses
- Increase in villus γ-H2AX staining after 90 days of exposure
- No increase in k-ras codon 12 GAT MF after 90 days of exposure (crypt & villus)
- Cr is localized to duodenal villi of mice *and rats*
- Cr in villi (alone) does not appear sufficient to cause duodenal tumors

Proposed RfDs

Thompson et al. (2014)



Research Article

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A chronic oral reference dose for hexavalent chromium-induced intestinal cancer†

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ABSTRACT: High concentrations of hexavalent chromium [Cr(VI)] in drinking water induce villous cytotoxicity and compensatory crypt hyperplasia in the small intestines of mice (but not rats). Lifetime exposure to such cytotoxic concentrations increases intestinal neoplasms in mice, suggesting that the mode of action for Cr(VI)-induced intestinal tumors involves chronic wounding and compensatory cell proliferation of the intestine. Therefore, we developed a chronic oral reference dose (RfD) designed to be protective of intestinal damage and thus intestinal cancer. A physiologically based pharmacokinetic model for chromium in mice was used to estimate the amount of Cr(VI) entering each intestinal tissue section (duodenum, jejunum and ileum) from the lumen per day (normalized to intestinal tissue weight). These internal dose metrics, together with corresponding incidences for diffuse hyperplasia, were used to derive points of departure using benchmark dose modeling and constrained nonlinear regression. Both modeling techniques resulted in similar points of departure, which were subsequently converted to human equivalent doses using a human physiologically based pharmacokinetic model. Applying appropriate uncertainty factors, an RfD of 0.006 mg kg^{-1} day^{-1} was derived for diffuse hyperplasia—an effect that precedes tumor formation. This RfD is protective of both noncancer and cancer effects in the small intestine and corresponds to a safe drinking water equivalent level of 210 μg l^{-1}. This concentration is higher than the current federal maximum contaminant level for total Cr (100 μg l^{-1}) and well above levels of Cr(VI) in US drinking water supplies (typically ≤ 5 μg l^{-1}). © 2013 The Authors. *Journal of Applied Toxicology* published by John Wiley & Sons, Ltd.

Supporting Information may be found in the online version of this article.

Keywords: risk assessment; hexavalent chromium Cr(VI); mode of action; benchmark dose (BMD) modeling; constrained nonlinear regression; cancer reference dose (RfD); intestinal cancer

Introduction

Exposure to hexavalent chromium [Cr(VI)] has long been recognized to increase the risk of lung cancer among workers in certain industries (IARC, 1990), as well as in rodents via inhalation or intratracheal instillation (Glaser *et al.*, 1986; Steinhoff *et al.*, 1986). Owing to protective reductive mechanisms, ingestion of Cr(VI) was thought to pose relatively little cancer risk (De Flora *et al.*, 1987; De Flora *et al.*, 1997; Febel *et al.*, 2001; Proctor *et al.*, 2002). In fact, Cr(VI) has not been shown to cause a significantly increased cancer risk in the alimentary canal of exposed workers (Gatto *et al.*, 2010). However, a recent 2-year cancer bioassay indicated that chronic exposure to Cr(VI), administered as sodium dichromate dihydrate, caused a dose-dependent increase in intestinal damage and intestinal tumor formation in B6C3F1 mice, but not F344 rats (NTP, 2008b). Subchronic bioassays indicated increased intestinal damage in mice after 90 days of exposure, but without evidence of preneoplastic lesions (NTP, 2007; Thompson *et al.*, 2011b). It is well known that chemicals that cause cytotoxicity and subsequently induce cell proliferation in shorter-term assays are often carcinogenic in longer-term bioassays (Ames *et al.*, 1993; Boobis *et al.*, 2009; Cohen, 2010; Gaylor, 2005). Thus, the disparate outcomes observed in mice and rats suggested that the intestinal tumors observed in mice

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- Rodent PBPK models were used to convert NTP 2-yr study doses into into internal Cr(VI) dose metrics
- Dose metrics for the duodenum, jejunum, and ileum of both sexes were combined to create a single robust dose-response curve
- Tumor or hyperplasia incidence were modeled using EPA's BMDS software
- BMDL values based on internal doses were derived and a 3-fold interspecies UF applied to account for possible differences in pharmacodynamics
- Human PBPK model was used to predict human exposure that results in internal dose equivalent to BMDL
- Applied a 10-fold human variability factor
- RfD = 0.006 mg/kg; DWEL of 210 ppb

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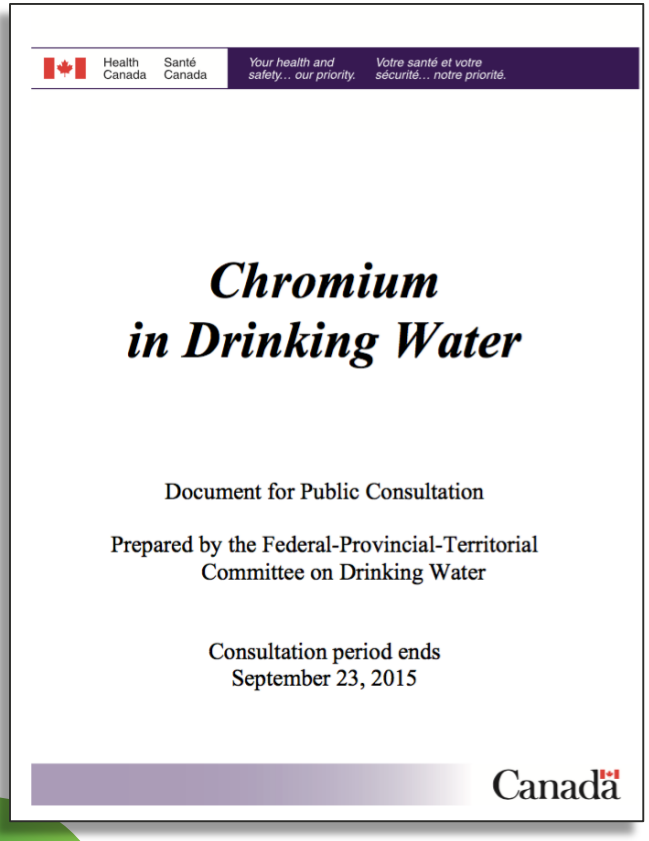
Health Canada (2015)



- Similar approach as Thompson et al. (2014)
- Human PBPK model was used to predict human exposure that results in internal dose equivalent to BMDL (different assumptions than used in Thompsons et al.)
- Applied 25-fold UF (2.5 interspecies; 10 intraspecies)
- TDI = 0.0044 mg/kg; DWEL of 100 ppb (after 50% contribution adjustment)

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TCEQ (2015)

- TCEQ value based on analysis published in Haney (2015)
- Modeled NTP hyperplasia data using 13-wk duodenal Cr levels from MOA research study (Kirman et al. 2012)
- Also modeled the relationship between duodenal levels and mg/kg bw dose
- Then converted the BMDL based on duodenal Cr levels to a mg/kg bw dose
- Applied 100-fold UF (10 interspecies; 10 intraspecies)
- RfD = 0.003 mg/kg; DWEL of 100 ppb ≅ MCL

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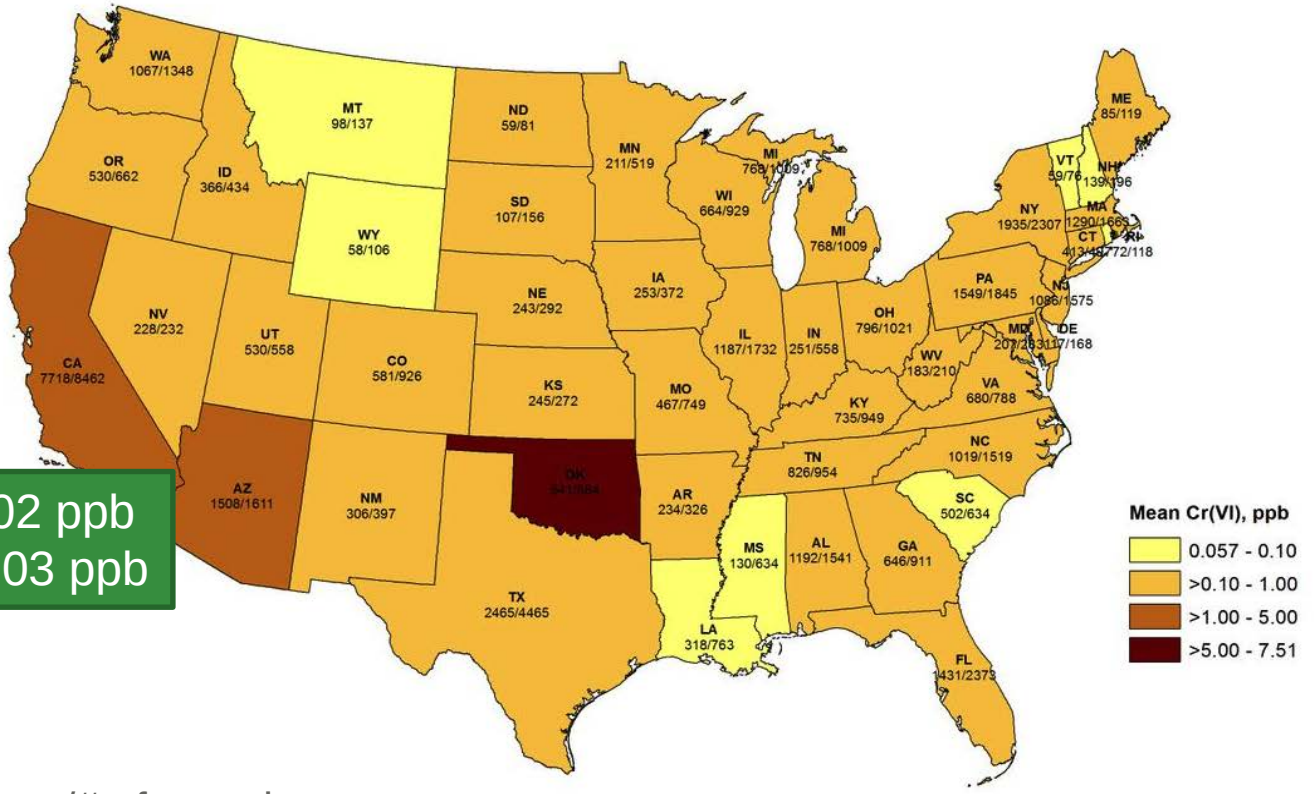
Summary of RfD Values Protective of Cancer

Source	RfD or TDI (mg/kg-day)	Drinking Water (ppb)	Data Used
Thompson et al. (2014)	0.006	210 (proposed keep MCL)	NTP data PBPK models MOA research
Heath Canada (2015)	0.0044	100 (increased from 50)	NTP data PBPK models MOA research
Haney (2015), TCEQ (2015)	0.003	≅ MCL	NTP data PK data MOA research

Cr(VI) Levels in Drinking Water

Average Cr(VI) Levels in U.S. (UCMR3, June 2015)

CA PHG = 0.02 ppb
EPA RSL = 0.03 ppb



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